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THE BIOLOGICAL APPROACH TO BACTERIOLOGY[1]

J. HOWARD BROWN

Department of Pathology and Bacteriology, Johns Hopkins University, Baltimore, Maryland

There probably is no scientific society, unless it be the chemical society, in which the membership represents a greater diversity of interest than does the membership of our society. Some of our members are interested in discoveries which may aid medical or sanitary science, which may alleviate suffering, help in the conquest of disease. Some are interested in the advance of agriculture, animal husbandry, the dairy industry, the fermentation industries or various other industries in which bacteria play a rôle. Much good bacteriological work has been done in the service of these special interests. In the popular mind practical discovery in applied bacteriology justifies the existence of bacteriology and certainly attracts to it considerable financial support. Of those who tread these avenues of approach to bacteriology it may be said that they are interested in what bacteria do rather than in what they are and more especially in some of the things that they may do. All of us are more or less guilty of having special interests whether we serve them or not. However, we have also a common interest which makes this society more than a federation of bacteriological workers and this meeting more than a composite of its various sections. It is for this reason that many of us are attracted to these meetings. We come here not merely to hear papers on medical, dairy or agricultural bacteriology, but rather to broaden our view of the whole field of bacteriology and to cultivate the acquaintance of our colleagues. Our common interest is bacteria, what they are as well as what they do. Some of us may be heretical enough to admit that we are less interested in sick patients, sick food, sick beer or big holes in swiss cheese than in the bacteria which cause these results.

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[1] Presidential address, delivered before the Society of American Bacteriologists at its thirty-third annual meeting, Baltimore, Maryland, December 28–30, 1931.

Diversity of interest does not necessarily imply diversity of motive. It is not inappropriate that I should share one of the prejudices of a fellow Baltimorean who has shown an appreciative interest in microbiology and the custodianship of some of whose yeasts I inherited from my predecessor at Johns Hopkins University, the former president of this society. Mr. H. L. Mencken writing of The Scientist says:

> The value the world sets upon motives is often grossly unjust and inaccurate. Consider, for example, two of them: mere insatiable curiosity and the desire to do good. The latter is put high above the former, and yet it is the former that moves some of the greatest men the human race has yet produced: the scientific investigators. What animates a great pathologist? Is it the desire to cure disease, to save life? Surely not, save perhaps as an afterthought. He is too intelligent, deep down in his soul, to see anything praiseworthy in such a desire. He knows by life-long observation that his discoveries will do quite as much harm as good, that a thousand scoundrels will profit to every honest man, that the folks who most deserve to be saved will probably be the last to be saved. No man of self-respect could devote himself to pathology on such terms. What actually moves him is his unquenchable curiosity—his boundless, almost pathological thirst to penetrate the unknown, to uncover the secret, to find out what has not been found out before. His prototype is not the liberator releasing slaves, the good Samaritan lifting up the fallen, but the dog sniffing tremendously at an infinite series of rat-holes. And yet he is one of the greatest and noblest of men. And yet he stands in the very front rank of the race.

It is in defense of those who enjoy "sniffing at rat-holes" that I speak this evening. Many who approach bacteriology from the medical or industrial avenue become inoculated with the virus of pure curiosity. Others, thrilled by the first view of the world of microscopic life, entering bacteriology from curiosity, but irresistibly drawn into industrial or medical bacteriology, may, nevertheless, retain that curiosity and may share the feeling of Pasteur who is reported to have said:

> If I have a regret, it is that I did not follow the route, less rude it seems to me, and which would have led, I am convinced to wonderful discoveries. A sudden turn threw me into the study of fermentation, fermentation set me at diseases, but I am still inconsolable to think that I have never had the time to go back to my old subject.

None will deny that bacteriology is a branch of biology. None would expect biological interest to be dominated by horticulture or animal husbandry. The arts of applied biology find their fertile fundamental concepts in the discoveries of pure biology. Similarly, applied bacteriology is dependent upon fundamental discoveries in pure bacteriology, whether made by botanists, industrialists or medical men. For such discoveries the biological approach is necessary. Bacteria themselves must be studied. What were some of the fundamental discoveries in bacteriology which made applied bacteriology possible? The discovery of the existence of bacteria; their form; their nature; methods and media for cultivating them; the method of excluding them by use of the cotton plug; methods of destroying them (sterilization); the existence and function of spores; methods of staining; the growth of colonies on solid media and the isolation of pure cultures; research on the so-called question of spontaneous generation (biogenesis or abiogenesis); the question of heterogenesis, xenogenesis or the existence of stable groups of bacteria; systematic bacteriology. These were fundamental biological studies in bacteriology. Without them, little or no subsequent progress could have been made. The first discovery that a microörganism might bring about such chemical changes as fermentation and putrefaction was a biological discovery of prime importance. Almost equally fundamental was the discovery that different kinds of bacteria produce different fermentations, the recognition of specificity. Less fundamental was the seeking out of the various organisms that bring about the different fermentations. Likewise, the first discovery that a microörganism was the etiological agent of an infectious disease, whether of man or of worm, was a biological discovery of far greater importance than the subsequent discovery of such specific pathogens as the tuberculosis bacillus, the typhoid bacillus or the diphtheria bacillus. I think that Pasteur's almost accidental discovery of the possibility of immunizing chickens against fowl cholera was a greater biological discovery than his subsequent immunizations against anthrax and rabies. However, his genius was revealed in the application to the latter discoveries of the correct observations and deductions from the fowl cholera experiments. These instances serve, in some degree, to illustrate the difference between the merit of the thing discovered and the merit of the act of discovery. A discovery of prime importance is sometimes made with little effort. A great deal of genius may be displayed and hard effort expended with relatively unimportant results. Scientific discoveries are the result of genius and luck in varying proportions. In the discoverer, we justly admire genius and perseverance. As to the merit of the thing discovered, it makes not a particle of difference whether the discovery was the result of personal effort or luck. The merit of a discovery in pure science depends upon its usefulness, either as a method or as an idea, as exerted on subsequent research. Judged by this standard, possibly Leeuwenhoek's observations and the demonstration of the usefulness of the cotton plug by Schroeder and Dusch were greater discoveries than that of the tuberculosis bacillus by Koch. History may be viewed either from the biographical viewpoint or by tracing the development of ideas, social movements, etc. To me, in the history of science, the dependence of one discovery upon another, the growth of science irrespective of the human instruments of discovery, is the more interesting view.

Tonight, I wish to call attention to the return of the purely biological interest in bacteria. Apparently bacteriology was born of three interests; the philosophical, the industrial and the medical. Perhaps these interests might better be called the biological, the industrial and the medical, biology lying well within the scope of natural philosophy. These avenues of approach are easily traced in the history of bacteriology. It is difficult to say whether interest in the origin and nature of minute living things, the cause and nature of putrefaction and fermentation or the cause and nature of contagious disease was first in time. Since the beginning of recorded history there have been many theories regarding these phenomena. The birth of bacteriology was delayed by the lack of a proper instrument, the microscope. There are three recognizable periods in the history of bacteriology. The first period was that of fundamental biological and technical discovery beginning with the first descriptions of bacteria by Leeuwenhoek in 1675 and extending to about 1885, the greater part of modern bacteriological technique having been developed during the last thirty years of this period. Since then there have been many improvements in technique but relatively few fundamental discoveries in technique,—among which, however, might be mentioned the ultra-microscope, the determination of reaction in terms of hydrogen-ion concentration, the use of ultra-violet light and methods of isolating single cells.

The second period may be described as that of the microbe hunters, beginning about 1849 with the discovery of the anthrax bacillus by Pollender and extending to about 1900. Undoubtedly, to those engaged in the search for specific organisms and to the world at large this was the most exciting period in the history of bacteriology. The technical and biological discoveries of the previous period bore fruit very rapidly. Certain industries and medical science were revolutionized. A noted bacteriologist of this period has said that it was difficult to wait for the morning newspaper to learn what pathogenic germ had been discovered. Within two decades, from 1880 until 1899, scarcely a year passed without the announcement of the discovery of the etiological agents of several long dreaded diseases against which mankind had been almost helpless. In 1892 John Tyndall said

> We have been scourged by invisible thongs, attacked from impenetrable ambuscades, and it is only today that the light of science is being let in upon the murderous dominion of our foes.

The light revealed not only the microbes of disease but also their mode of transmission, means of avoiding infection and, in some instances, means of overcoming infections. There were revealed not only the hidden enemies of man but also his microscopic friends that henceforth could be made to serve more efficiently in the preparation of food, drink, clothing and chemical products as well as in the fertilization of the soil. The industrial revolution brought about by bacteriology can be compared to that wrought by the use of electricity. Much of the bacteriological work of this period was inspired by medical and industrial interest. Biological interest in bacteria was rather incidental. Although advance in systematic bacteriology continued, the dominant school of medical bacteriologists was rather impatient with such attempts and it is still difficult to interest them in the biology of non-pathogenic bacteria. Even Pasteur cared little about the morphology of bacteria and less about what they might be called or their relation to each other but was intensely interested in their activities in so far as they affected human welfare. A period of too exclusive devotion to practical results is self-limiting in any branch of science. Bacteriology began to slow up a pace. It came of age. There were still etiological agents of disease and industrial applications to be discovered but they did not readily yield up their secrets to application of the old knowledge and technique.

The period of modern bacteriology began slowly to dawn. Many of us who began to work at this time felt the difficulties that were encountered. We looked back with envy upon the spectacular period that had been written so recently into our textbooks. The older methods were no longer sufficient. It became necessary to dig deeper into chemistry and biology. The foundations of bacteriology needed broadening. Bacteriology ceased to be merely a tool of industry and pathology and began to reappear as a separate branch of biology, the subject matter of which was not disease or fermentation but bacteria. This society and its journal represent this trend. We hear less about recently discovered etiological agents and more about biological and cultural phenomena such as bacteriophage, microbic dissociation, growth phases, the physiology of bacteria, the possibility of the existence of life cycles and of filterable forms of bacteria. These are all recent fundamental biological discoveries and problems. Incidentally these phenomena may have practical significance, such as relations to virulence and immunity, but the fundamentally important considerations are the existence and nature of the phenomena. Bacteriology is not alone in showing a tendency to adopt a more purely biological viewpoint. Immunology has become quite as much concerned with the relation of bacteria to each other, the chemical constitution of the antigenic constituents of the cell, and the physico-chemical mechanism of serological reactions as with immunity or recovery from disease. Pathology and clinical investigation are biological sciences as; in fact, is all medical science as distinguished from medical practice. There are indications of a growing tendency to approach medical science from the biological rather than the therapeutic avenue. I have been much impressed by the following passage from the recent presidential address of Dr. F. G. Blake before the American Society for Clinical Investigation.

> I believe it should be kept in mind that the purpose of this study of disease should be primarily to find out about disease, largely for the fun of doing it, to discover the circumstances or conditions under which disease develops, the nature and mechanism of the disturbances of function and structure which take place during the course of disease, and the circumstances or conditions under which recovery or death occur. Secondarily, this may lead, and fortunately sometimes will, to the discovery of methods of prevention, amelioration or cure, but these practical and humane purposes should, I believe, be kept in the background, if clinical investigation is not to be too soon diverted and frequently misled in following its main purpose, the elucidation of the phenomena of disease.

More startling, perhaps, is the following which Dr. Blake quotes from Slesinger on "The Drift of the Social Sciences."

> Social science shares with medical science the necessity of having to free itself of the desire to do good and of measuring its success by the amount of good accomplished. The medical sciences are only recently beginning to abandon the therapeutic aim in research. It is not to be wondered at, therefore, that the younger social scientists find themselves still slightly hampered by an attitude growing not out of this subject matter, but out of the personnel attracted to the field during a

stage in the development of research when welfare was more important than truth.

I might add that I do not think that these quotations imply that there is not a science of therapeutics but the aim of this science should be to find out what may influence the course of disease favorably or unfavorably; why certain reactions follow certain treatment. The desire to do good may quite properly motivate the practice of medicine or the application of science in general but there is a danger in allowing any other motive to enter into scientific research than that of finding out, of discovering truth. A humane desire or an industrial need may indicate where research is needed but during the progress of research such desires are best forgotten. If such desires motivate the research, the researcher becomes a prospector rather than an explorer, an inventor rather than a scientist. In students it is most important to cultivate healthy skepticism and a critical faculty. They are impatient with details, anxious for practical results, eager to read only the latest publications. Just as it is unsafe to build a super-structure of theory upon too few facts, there is a double danger in the too eager application of new discovery to practical ends. There may be danger to man himself, as illustrated by the tragedy of the therapeutic use of tuberculin too soon after its discovery and possibly by too great confidence in the avirulence of rough dissociates of pathogenic bacteria used for immunization. There is also the danger that fundamental research may be arrested by attention being diverted to its practical application. It would be unfortunate if interest in bacteriophage should be abandoned because it has not been found to be a panacea for disease. Science can wait and cares little for the individual or for this generation of individuals in preference to the next.

The drift of the social sciences, medical science and bacteriology to biology reminds me of the drift of all science towards greater exactness as pointed out by Professor Bowman in a series of lectures on philosophy delivered some years ago at Princeton University. Professor Bowman, as I remember it, stated that as the several sciences become more mature, social science drifts towards and tends to find explanations to interpret its phenomena in terms of biology; biology, in like manner, drifts towards chemistry; chemistry towards physics; and physics towards mathematics.

When one arrives at mathematics there seems but one other thing to do—to philosophize. We are justified in asking—If the desire to promote human welfare, to do good, is to be excluded from scientific research, what is its justification? The trite answer is "truth for truth's sake" just as art may be justified for art's sake. As a proximate answer this may serve very well but actually it is no answer at all. A man may do research for the fun of doing it but he can not expect to be supported for the fun of doing it. After all, truth and art are human concerns and have no justification and probably no existence outside of the mind. Pontius Pilate's question, "What is truth?", was not answered. Herbert Spencer stated that

> what we call truth, guiding us to successful action and the consequent maintenance of life, is simply the accurate correspondence of subjective to objective relations; while error, leading to failure and therefore towards death, is the absence of such accurate correspondence.

This definition of truth seems to me to contain the whole of the pragmatic philosophy. The motive of those who sniff at rat-holes is curiosity and the fun of doing it. Their reward is curiosity satisfied. Their social justification is that experience has shown that this motive most effectually excludes prejudice and most successfully contributes to a body of truth which enables man to make that "continuous adjustment of internal relations to external relations" which is Spencer's definition of life.

In trying to emphasize the importance of the biological approach to bacteriology I have not meant to speak disparagingly of research undertaken for solving practical problems. We need not be ashamed of being prospectors or inventors at times. Even Mr. Spencer invented "A New Invalid-bed" and "A New Fishing-rod Joint." In speaking of the biologist I refer not to individuals but to any of us in what may be called our biological moments, those moments when we are actuated by purely scientific motives. At such times the medical man and the industrial bacteriologist are biologists. It is because bacteriology is a biological science of which the chief concern should be bacteria and because the biologist is relatively free from those praiseworthy but prejudicial motives which may influence those who are interested primarily in applied science that I view with gratification what seems to me to be the renaissance of the biological approach to bacteriology.

THIRTY-THIRD ANNUAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

BALTIMORE, MD., DECEMBER 28, 29 AND 30, 1931

Headquarters: Lord Baltimore Hotel

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ABSTRACTS

Report of Committee on Bacteriological Technic: Progress During 1931. H. J. CONN, *Chairman;* VICTOR BURKE, IVAN C. HALL, J. A. KENNEDY, BARNETT COHEN, ELIZABETH F. GENUNG, *Members.*

The Committee does not have a great deal of activity to report this year largely because of a six months' leave of absence on the part of the Chairman. Routine matters, however, have been progressing in a satisfactory way and important plans made for 1932.

The work of revising the Manual of Methods for Pure Culture Study has been completed during the year, and it now consists of a series of nine leaflets, each dealing with some general phase of methods. It is planned to revise the leaflets often enough to keep them up-to-date. At present Leaflet VII of the series is missing. This is to deal with methods used in the isolation of pathogens and determination of pathogenicity. Manuscript for this leaflet, however, written by one of the committee members is now almost complete and it is expected to publish this leaflet early in 1932.

The Manual continuation service through which holders of copies of this Manual arrange to have all new material sent to them as issued is at present in a very flourishing state. An unusual number of new subscriptions have been received this year and the list has now reached nearly 600. It was announced last year that it was hoped shortly to put this service on a periodical basis, issuing new material regularly once a quarter. Plans for this change are now nearly complete. It is felt that this change will add to the convenience of the subscribers and at the same time will make the distribution of the revised leaflet more economical.

Coöperation of other societies through the Commission on Standardization of Biological Stains is still continuing. This work is at present forced to operate on a reduced appropriation from the Chemical Foundation. This has been done up to the present without curtailment of the Commission's activities, because other sources of income have been drawn upon. It may some time be necessary to ask some financial assistance from the societies coöperating in this work. At present, however, no such assistance is required, and efforts are being made to put the work as nearly as possible on a self-supporting basis.

Report: The Recent League of Nations Conferences on the Serology of Syphilis. R. L. KAHN, University of Michigan, Ann Arbor.

Within recent years, the Health Organization of the League of Nations held two competitive serological conferences, one at Copenhagen (1928) and the other at Montevideo (1930) with a view to determining which well-known tests for syphilis stood out in specificity and in sensitivity. The dependability of 7 different Wassermann modifications and 7 precipitation methods was tested at the Copenhagen Conference, and of 7 Wassermann modifications and 5 precipitation methods, at the Montevideo Conference. Approximately 1000 sera were examined at each of the conferences with these methods which were performed by their respective authors in practically all cases. The Health Organization reports of these two conferences are now available, making possible a summary of the results. The Kahn reaction which at both conferences "proved to be absolutely specific and extremely sensitive" is considered in relation to the other methods. The far-reaching importance of these conferences is discussed.

Report: The Work of the Nomenclature Committee of the International Society for Microbiology. ROBERT S. BREED, NEW York State Agricultural Experiment Station, Geneva.

At the first International Congress sponsored by the International Society for Microbiology in Paris 1930, provision was made for the appointment of an International Committee on Bacteriological Nomenclature. At the International Botanical Congress held in Cambridge, England, a few weeks later, the arrangements made at Paris were approved and plans made for coöperative work in this field.

The personnel of this International Committee has been partially completed through the appointment by the National Committees of the following: Hungary, *Aǔjeszky*, *Manninger;* Roumania, *Cantacuzène*, *Ionescu-Mihaiesti*, *Zotta;* Bulgaria, *Petroff*, *Markoff;* Germany, *Lehmann*, *Reiner-Müller;* Holland, *Flu*, *Aldershoff*, *Kluyver;* Denmark, *V. Jensen*, *M. Christiansen*, *Orla-Jensen;* Sweden, *Petterson*, *Hülphers*, *Barthel;* Russia, *Issatchenko;* Austria, *Janke;* Spain, *Murillo*, *Falco;* Italy, *di Rossi*, *Azzi;* Great Britain and Ireland, *P. B. White*, *J. Ramsbottom*, *W. M. Scott*, *A. C. Thaysen*, *R. St. John-Brooks*, Permanent Secretary, Medical and Veterinary Bacteriology; United States, *R. E. Buchanan*, *O. Rahn*, *E. Pribram*, *I. C. Hall*, *J. H. Brown*, *R. S. Breed*, Permanent Secretary, Agricultural and Industrial Bacteriology; Switzerland, *Doerr*, *Frei;* Poland, *Eisenberg*, *Szymanowski;* Canada, *W. L. Holman*, *G. B. Reed.*

Without waiting for all of the appointments to be made, plans have been made for submitting problems in bacteriological nomenclature to this International Committee for their action in order that some decisions may be ready to submit to the next Congress held under the auspices of the International Society of Microbiology (Berlin, 1933). It is recognized that this Committee does not have power to enforce any of its decisions but it is hoped that the vote of this group may be founded on such accurate information that bacteriologists generally will feel justified in accepting their decisions.

GENERAL BACTERIOLOGY

Symposium on Bacterial Dissociation and Life Cycles

G1. Phases of Growth in Size of Bacilli and Yeast Measured from Motion Pictures. Stanhope Bayne-Jones and Edward F. Adolph, Departments of Bacteriology and Physiology, School of Medicine and Dentistry, University of Rochester, N. Y.

The object was to measure the rates of growth of single individuals and their progeny throughout a cultural cycle.

Individuals of *Bacillus megatherium* and *Saccharomyces cere-*

visiae were implanted on the surface of a small cone of 2 per cent peptone agar. These were photographed two to thirty times per minute for several hours at constant temperature. From projections of single frames of the film, linear dimensions were measured at frequent known intervals of time. Body volumes were calculated for bacilli as length (l) × square of breadth (b) × $\pi/4$, and for yeast as $l \times b^2 \times \pi/6$.

In the bacilli the breadth did not vary measurably during one generation, and the length increased with constant percentage increment (hence logarithmically). There was no measurable slowing while fission was being prepared for or going on. In the yeast, on the other hand, the increment waxed and waned with each generation, being slowest just before a new bud formed.

During the period of most active multiplication, both the mature sizes of individuals and the duration of one generation exhibited normal distributions. After 3 hours' cultivation at 37°C. *B. megatherium* had a mean adult volume of 24 cubic micra and a mean generation time of 26 minutes; yeast, 97 cubic micra and 74 minutes.

Cultural cycles were exhibited most markedly in *B. megatherium* Under the particular conditions of culture used, after implantation from a 24-hour-old broth culture the lag phase lasted 2 hours, the phase of logarithmic increase prevailed from this time until 4 hours, and during the fifth hour the rate of growth suddenly declined almost to zero. The mean volumes of the adult individuals slowly increased during the first four hours of culture, thereafter they decreased. The increment of total bulk in the culture was proportional to the rate of growth of the individual.

In yeast also the age of the culture had less influence upon adult sizes than upon the rates of growth. The concentration of nutriment and the temperature affected markedly the rates of increment in mass.

G2. Bacterial Variation. LEO F. RETTGER AND HAZEL B. GILLESPIE, Department of Bacteriology, Yale University, New Haven, Conn.

The present investigation is, in a sense, a three-cornered one, dealing with (1) pleomorphism, (2) structure of the bacterial envelope, and (3) filterability of bacteria.

B. megatherium lent itself admirably for the study of wide variation in morphology as influenced by environment. The cellular morphology varied with the medium employed, and with the temperature and duration of incubation. When grown on a meat extract agar at 22°C. for from 24 to 48 hours, the usual picture was obtained in the slide mount, the cells being more or less uniform and occurring in short and long chains. However, when incubation was at 37°C., very unusual forms appeared at the end of 48 hours. There were many shadowy (autolyzed) bacillary types possessing prominent granules. There was also a variety of deeply-staining filaments which were often distinctly clubbed at one end.

When grown on skim milk casein digest agar at 22°C. for 48 hours an entirely different microscopic picture was presented. Very few so-called normal cells were seen. Variation in morphology occurred within a very wide range, but the outstanding types were the boat-, spade- and short club-shaped forms of varying sizes. An entirely different picture was again obtained in cultures which had been kept on ordinary meat-extract agar for at least 2 or 3 weeks. Here filaments of varying length and thickness, often showing true branching, were the outstanding feature.

Repeated observations have shown *B. megatherium* to possess a tough skin-like cell wall. Cells which had autolyzed, with apparently nothing but the covering left, appeared like flattened sausage skins or baby ribbon. This was observed best in cells that had folded over on themselves. This wall-like structure was made apparent also in cells the tips of which had been partially torn away. Furthermore, the teasing of individual cells with a micro-needle showed them to be tough and elastic. Long filaments could be broken only by bringing considerable pressure on them with the needle. Work is under way to follow the growth of individual cells, both the extreme variants and the ordinary type. Thus far our efforts have met with success in only 3 instances.

The laboratory has been engaged for almost 2 years in attempts to demonstrate the occurrence of filterable forms of several strains of Brucella, and of one of the stock strains of *B. megatherium* used in the pleomorphism experiments: also of several strains of *B. mesentericus* and *B. vulgatus* studied by Miss Flynn. We have not as yet been able to demonstrate filterability of the organisms employed under experimental conditions involving a system of adequate control.

G3. Bacterial Mutations. F. d'HERELLE AND RUTH BEECROFT, School of Medicine, Yale University, New Haven, Conn.

Transformation in the characters of bacteria is a phenomenon which has been noticed since the early days of bacteriology; but it has particularly attracted the attention of scientists in the last ten years. All who have studied this problem have worked with bacterial strains obtained by chance from collections, retaining the one which could be "dissociated" and discarding others as unsuitable for the purpose. Such a method of approach to the problem of bacterial transformations cannot lead to any valid conclusion, as the cause of the transformations cannot be detected.

In 1918 I showed that bacteria undergo profound transformations under the action of a phage, and since that time I have continued to study this phenomenon in relation to various species of bacteria.

During the three last years I have made a special study of a strain of *Salmonella typhi-murium* and have reached the conclusion that ultrapure (phageless) strains of bacteria do not show any modifications and they remain stable through repeated passages (more than 500) on various artificial media.

Contamination of an ultrapure strain with a phage gives rise to mutating strains, which remain phage-carriers indefinitely. From the moment of the contamination, mutations occur in every direction and each of the characters varies by itself as a separate entity with a result that the number of possible mutations, all from a single primitive bacterium, is indefinite.

All the peculiarities of the phenomenon indicate that bacterial transformations are not due to a "life-cycle" but are true mutations, similar to those which have been described by botanists as occurring in plants under the action of parasitism. Bacterial transformations are a particular case of an already well known phenomenon, that of mutations under the action of a symbiosis.

G4. *Some Observations on the Quantitative Occurrence and the Mechanism of Formation of Induced Bacteriophage.* C. N. STARK, P. W. STARK AND RUTH GORDON, Cornell University, Ithaca, N. Y.

Bacteriophage has been produced from three different stock cultures of *Bact. aerogenes* which had never been known to exhibit any phagic action. The phage was developed in each culture without the addition of any unsterile material or filtrates of extraneous matter.

Quantitative estimates of phage by previous workers have indicated its presence in "numbers" varying from 10^7 to 10^{13} per cubic centimeter, most determinations showing about 10^8 to 10^{11}. In our work with *Bact. aerogenes* estimates of phage have repeatedly shown it to be present in numbers of 10^{12} per cubic centimeter, as indicated by complete clearing of broth cultures. As measured by plaque formation on agar, the indicated number of phage units reached 10^{13} per cubic centimeter.

Our results very definitely indicate that the phage increases during the lysis of the bacteria.

G5. *New Studies on the Filtrability of Pure Cultures of the Tubercle Group of Microörganisms.* RALPH R. MELLON AND L. W. FISHER, Institute of Pathology, The Western Pennsylvania Hospital, Pittsburgh.

The experiments to be reported here have been designed to overcome the last remaining obstacles to the acceptance of the view that the acid-fast group of bacteria possess a filtrable phase in their life cycle. The matter is stated in this way because as it happens we have employed chiefly a timothy bacillus rather than the tubercle bacillus, although even with the latter, complete parallelism exists as far as we have gone.

The organism made the subject of these experiments was isolated in 1925 as an acid-fast contaminant in other work. Attention was called to it because it grew as did the tubercle bacillus, and on closer examination was found to be acid-fast and indistinguishable morphologically from this organism. Because at certain times its acid-fastness was quite variable, it was single-celled for purity, but without effect on this particular variability.

On several occasions during 1925–1927 we took advantage of the presence of the gonidial phase by filtering it. These gonidial granules developed under conditions that will be described somewhat more in detail. The filtrates on these occasions germinated as a non-acid-fast diphtheroidal form, culturally bearing no resemblance to the original, but microscopically identical. At this time the original form did not come out of the filtrates.

This strain has been under observation for six years since its isolation and four times, always in the spring of the year, spontaneous dissociation has occurred without filtration. The dissociant was identical in cultural characters with the strains previously obtained in the filtrates. A similar strain has been obtained from a pure culture of a bovine tubercle bacillus. Although growing in fine translucent colonies on blood agar, it frequently contains large numbers of acid-fast granules, some of which appear to be extracellular.

Recently by employing a modified form of the K-medium (Kendall) we have repeatedly obtained similar cultures from the filtrates differing only from the ones previously isolated in greater range of characters of the individual strains. Strains identical with those recovered from the original filtrates, as well as strains representing a complete reversion to the original grass bacillus, have been grown from the filtrates. Also intermediate stages between these extremes.

These cyclostages possessed of acid-fast granules are quite similar to those isolated from Hodgkin's disease and benign lymphogranulomatosis (Schaumann), suggesting their in vivo origin, but without vouchsafing any rôle for them in the pathogenesis of the disease.

Summed up, the links in the chain of evidence are as follows:

first, demonstration that the acid-fast gonidia of a granular (not bacillary) sputum pass the filter (Luksch, 1930); second, although greatly attenuated they will cause typical tuberculosis from which the virulent organism may be recovered (Mellon, 1927; Panek, 1930); third, that timothy bacilli in pure culture form gonidia which will germinate into the original strain, or one or more intermediate non-acid-fast diphtheroid strains; fourth, that identical diphtheroid strains dissociate spontaneously without filtration; fifth, that diphtheroids with abundant acid-fast granules dissociate from pure cultures of bovine tubercle bacilli, their origin being presumably the granules of Much.

G6. Observations on the Life Cycle of Acid-fast Bacteria. MORTON C. KAHN, Cornell University Medical College, New York City.

An investigation on a single, living bacterium showed in part that *Mycobacterium tuberculosis*, strain H37, did not multiply by simple fission under our conditions, but underwent usually a complicated developmental cycle, the more important phases of which were cleavage of the rod into three or more ovoid units, formation of these units into diplococcoid bodies, and subsequent division of these diplococci into minute oval bodies, which later become reduced to a state of dust-fine material from which extremely small and delicate rods developed. Later, these tiny rods, by a process of elongation and thickening became what may be called the mature tubercle bacillus. Differences in the staining reaction of various components of the cyclic scheme were also noted. Some of these were found to be non-acid-fast. These phenomena were observed repeatedly and there seems to be little question as to the sequence of events at least under our experimental conditions. These investigations of the life cycle were then extended to the avian *M. tuberculosis* using the technic elaborated for the single cell studies of the human strain. This, very briefly, consists in the use of the Chambers micromanipulator and a micro-pipette. Exceedingly small microdroplets are placed on the underside of scrupulously clean cover slips, a number of which, with proper dilutions of culture and other controllable factors, will be found to contain a single bacterium or small groups of from 2 to 6. The cover slip is then sealed in place upon a hollow ground slide and observations may thus be made on an identical organism for several days or weeks.

These studies on the avian organism were well under way when Petroff published his studies on the successful dissociation of the A-1 culture of the avian type as well as the B-1 and BCG strains of the bovine type. Upon applying Petroff's technic for dissociation to our culture of the avian organism, we found that it also dissociated into the R and S types. It was at once decided to see if dissociation played a rôle in the life cycle of this organism. Since there are many skeptics among workers on the tubercle bacillus as to whether or not dissociation is a *bona fide* phenomenon or a chance symbiosis of two distinct strains, one developing when conditions are favorable and the other being repressed when conditions are the reverse, it was necessary to begin with a single organism and to see whether cultures grown from this one cell could be induced to mutate into the R and S types. A single Rough A-1 avian organism was allowed to develop into a microscopic colony. When growth had spread over most of the surface of the microdroplet, a sterile micro-pipette containing sterile culture medium removed the entire growth and expelled it upon a slant of egg medium.

In 9 days a tiny, typically Rough colony developed. Several subcultures on egg medium plates made it evident that the colony offspring from this culture tenaciously maintained the original rough configuration. Attempts to induce smooth types to develop from this growth by altering the pH of the medium and by other methods were unsuccessful.

When, however, the R organism was injected intravenously into rabbits in 0.5 mgm. doses and cultures were made from the organs after death, it was found that typical S colonies developed from the marrow when plated out upon suitable media, although growth from other organs, namely, liver, lung, spleen, inguinal and axillary lymph nodes, gave only the typical R forms of colonies. This had previously been noted by Doan but with a non-single-cell strain. There was still a remote possibility that these S colonies may have been present in the animal prior to the introduction of our single cell strain. Therefore, experiments were made to induce dissociation without the use of an animal. Our single cell cultures were grown in glycerol broth containing 10 per cent of fresh normal rabbit serum which was proven to be sterile by rigid laboratory control. After 10 transfers on this medium, not a few S colonies appeared when the fine surface membrane was planted upon egg medium. About this time it was noticed that an acid-fast organism isolated by Chapin from the rat in 1912 and designated by him as a bacillus concerned in rat leprosy, had undergone spontaneous dissociation after having been incubated for several months. Typical R and S colonies were obtained from this single cell culture whereas the original growth had been entirely R in character. We applied this information to a single cell culture of the avian bacillus which had been incubating for 9 months and found that although this came from a single rough organism whose sub-cultures were typically R in character, material from the 9 month old culture when planted on egg medium gave rise to typical S as well as typical R colonies. That is, spontaneous dissociation had taken place during the long incubation of this initial R culture. This must be taken into consideration in any study of the life cycle of an organism for which dissociation has been shown. Our evidence leads us to believe that the factors which determine dissociation may be present in the germ plasm of each individual cell. Whether the organism grows as an R type or an S type must be governed by a set of determining laws with which we are only faintly familiar at present.

The R and S types show a number of distinctive features,—differences in the morphology of the rod, in the configuration of the colony, in a number of physical properties of the colony, including, as we have shown, differences in electrophoretic velocity, and as has been shown by Richardson, Loebel, and Shorr, differences in respiration. S cultures of the avian bacillus which are offspring of single cells may be made to mutate into the typical R type with a resultant diminution of pathogenicity; while the S mutant from a single-cell R culture reveals the enhanced pathogenicity of the typical S form. Our tests were made by inoculating intravenously into rabbits. Evidence supports the view that there is also a difference in the life history of the R and S types when they are in their respective phases. It seems, therefore that in investigating the life history of this group of organisms, the mutation cycle, as well as the life cycle of the individual rod must be considered.

In our opinion, the possibility of dissociation will sooner or later have to be regarded as part of the interpretation of the epidemiology and pathology of infectious diseases, including, at present, avian and bovine tuberculosis.

G7. *Some Observations on Variation of Bacterium dysenteriae, Sonne Type.* R. B. Dienst and S. A. Koser, University of Chicago, Chicago, Ill.

A small colony variant similar to the G type of Hadley was obtained from an ageing culture of *Bacterium dysenteriae* Sonne in casein digest broth. This variant was first seen on agar plates made from the casein digest after incubation for 30 days at 37°C. and after two previous platings had been "sterile."

Of approximately 40 cultures studied in a similar way or subjected to other procedures calculated to enforce variation, this was the only instance in which this small colony variant was found, although Sr, sR and R forms were usually in evidence after 7 to 14 days at 37°C.

Further study of the small colony variant showed that it could be subcultured in broth or on agar plates. The colonies usually attained a diameter of about 100 microns after 48 hours at 37°C. The morphology of the individual cells composing these small colonies presented a varied picture consisting of normal appearing rods together with club-shaped, swollen forms and long filaments. Sugar fermentation was qualitatively the same as with the normal parent strain but much slower. A number of filtration tests were made with this small colony variant, but all attempts to cultivate it from the filtrate have been unsuccessful.

This "G" form was brought back to the normal-sized colony

(S or Sr) by serial transfers in dextrose veal infusion broth and it then presented the typical morphological, biochemical and agglutinative reactions of the original parent strain.

G8. On the Antigenic Relationship of the Rough and the Smooth Variants of B. typhosus. FRANCIS B. GRINNELL, Harvard University Medical School, Boston.

That vaccination with rough strains, not only of *B. typhosus* but of many other organisms, is ineffective or at least inferior to vaccination with smooth strains in immunizing against subsequent infection with virulent cultures of the homologous organism is the conclusion which must be drawn from the extensive experimental evidence in the literature of bacterial dissociation. Rough strains of *B. typhosus* are, however, still very generally used in the production of anti-typhoid vaccines.

As the most direct practicable approach to the problem of assessing the possible difference in the degree of protection against typhoid fever in human beings produced by the rough and the smooth forms of *B. typhosus* when used as antigens for the customary course of prophylactic vaccination, bactericidal tests were carried out on the blood of individuals before and after such a course of vaccination.

The technique of the bactericidal tests was as follows: To 0.5 cc. of fresh defibrinated blood in each of a series of small Pyrex tubes was added one drop of progressive dilutions of an 18-hour culture of a smooth strain of *B. typhosus*. The tubes were then sealed and placed in a rotating box in the incubator. After incubation, a loopful from each tube was plated out to determine whether growth had taken place.

Such bactericidal tests were carried out on the blood of 8 individuals; 3 of these then received the customary course of 3 subcutaneous injections with a smooth vaccine, and 3 a similar series of injections with a rough vaccine. The other two served as controls on the virulence of the cultures used in the bactericidal tests. The average number of organisms killed by the blood of the individuals receiving the smooth vaccine was found to have increased 150 times, whereas the average number of organisms killed by the blood of those receiving the rough vaccine was slightly less after vaccination.

Whereas the rough vaccine proved ineffective in increasing the bactericidal power of the blood, the two vaccines were quite comparable in agglutinin production, since the agglutination was of the flocculent type due to the flagellar agglutinogen present in both vaccines.

Since the bactericidal tests obviously do not provide a complete index of the resistance of the individual to infection, the human experiments were supplemented by protection tests on mice which had received 3 intraperitoneal injections of the two types of vaccine. These experiments confirmed the results of the bactericidal tests. The mortality of the smooth vaccine group was considerably lower than that of the controls, whereas the mortality of the rough vaccine group was somewhat higher than that of the controls.

Cross-agglutination, agglutinin absorption and bactericidal tests with the sera of rabbits highly immunized by the repeated intravenous injection of the motile smooth, non-motile smooth, motile rough and non-motile rough variants of *B. typhosus* showed not only that the four forms contain a common somatic agglutinogen, but that under these conditions the rough strains are capable of inducing the formation of antibodies bactericidal to the virulent organisms.

The results of the bactericidal tests with rabbit serum are obviously not in agreement with the human experiment; but the results were clean-cut in both cases and although the reason for the contradictory results is not as yet clear, the writer is inclined to believe it due to the difference in degree of immunization.

G9. Dissociation and the Epidemic Problem—Some Observations from the Study of Influenza. I. S. FALK, Washington, D. C.

The literature and the etiology of the 1928-1929 pandemic of influenza indicates: (1) that the hemophilic bacilli typified by Pfeiffer's bacillus were apparently not responsible; (2) there was no positive evidence to incriminate "filterable viruses;" (3) that many investigators are agreed cocci played a prominent rôle; and at least two groups who studied the outbreak specifically incriminated cocci or streptococci.

Although a common etiology for different epidemics of influenza is not established, the assumption of etiological identity in different outbreaks is a doctrine of convenience. The experiments of Falk, Harrison, McKinney and Stuppy (Amer. Jour. Hyg., Monograph No. 11, Jan. 1931) are briefly reviewed and attention is focussed upon the problem of spontaneous changes in virulence which they observed in bacteria in culture. When the virulence of primary cultures declined so that after inoculation the influenzal disease was no longer incited in monkeys, dissociation was practiced. Virulence was found to vary with colony form. Subsequently—in a non-epidemic period—when identical or similar cultures were isolated from the throats of "normal" persons or of individuals affected with mild respiratory affections, these microbes were found to display the colony forms formerly found associated with the avirulent or only slightly virulent state. The significance of these findings for the relations between epidemic and inter-epidemic influenza and for "the epidemic problem" generally are briefly considered. Attention is focussed on "dissociation" in vivo, for in the study of this phenomenon may lie the heart of "the epidemic problem."

G10. The Changing Bacteria. C.-E. A. WINSLOW, Department of Public Health, Yale School of Medicine, New Haven, Conn.

The remarkable researches of the last ten years have made it abundantly clear that older concepts of the simplicity of the bacterial life cycle must be profoundly modified. Two distinct types of variation have been demonstrated. Both types are markedly affected by environmental stimuli, particularly by the presence of bacteriophage. Both are discontinuous and both definite and specific in nature. The first type may be called cyclogenic variation. It supplements our concept of the spore with the concept of the gonidium and the filterable phase, as essential phases of the life cycle of many bacterial species; and it seems to include the probability of a sex process in certain instances. This type of variation brings the bacteria into line with the higher fungi in their reproductive processes.

The second type of variation may be called dissociative variation. It involves the production by a wide variety of bacterial species of two or more alternative types characterized by sharp discontinuous differences in colony formation, biochemical activity, virulence and other characters. These variations suggest an analogy with mutations among the higher forms of life but they differ sharply from mutations in the fact that the changes seem generally reversible. The relation between cyclogenic and dissociative variation is as yet uncertain.

These new discoveries do not negate bacterial specificity but merely show that the characteristics of each bacterial species are vastly more complex than we had realized. They do not invalidate the bases of epidemiology; but they do give promise of solving some of our most puzzling problems of epidemiology and immunology. They have revolutionized the theoretical foundations and multiplied the practical possibilities of bacteriology.

G11. Filterable Migrant Forms of Mammococcus. COSTANTINO GORINI, R. SCUOLA SUP. Agricoltura, Milan, Italy. (Read by title.)

The direction of my bacterial researches led to examination of the physiologic dissociation of bacteria, which I showed, for the first time I believe, in 1921, while investigating the physiological mutation of *Bacillus acidificans presamigenes*, an acido-proteolytic variety of the Subtilis group (see review in Journal of Dairy Science, 5, September, 1922). In subsequent work (C. R. Ac. Sc., **190**, Janvier, 1930; Rend. Ist. Lomb. Sc. Lett. **64**, 1931, p. 393) I have shown physiologic dissociation also among streptococci and micrococci of the udder (*Mammococcus*), whose heterogeneity I described in 1902 (Rend. Ac. Lincei, **11**, Septembre, 1902).

I now wish to mention an interesting and suggestive case of morphologic dissociation of *Mammococcus*.

I had filtered a clotted milk culture of the acidoproteolytic *Mammococcus* through a sterile Pasteur filter in order to ascertain chymase in the filtrate. Fresh, cautiously sterilized milk added to the filtrate in the ratio 1:10 coagulated at 35°C. in 24 hours; methylene blue stains revealed no cocci, but a multitude of very small blue-staining slightly reddish granules. Transfer of one loopful from this clot in sterile milk did not coagulate, and did not yield cocci even after 14 days' incubation; but it yielded the above mentioned granules. This is a proof that the filtrate of the stock culture contained soluble rennin. But this enzyme is produced by the non-filterable cocci and not by the filterable granules. By repeated plating on ordinary peptone agar there developed two kinds of colonies: ordinary rough colonies of the non-filterable coccus and small tiny dew-drop colonies of filterable granules. The coccus colonies were able to coagulate milk. On the other hand, the granule colonies did not coagulate milk. The coccus colonies could readily be retransformed to the filterable state by transfer into milk refiltered and recovered again by plating on agar. Watery colonies could reinduce the non-filterable forms slowly after repeated cultivation upon ordinary agar or broth. Finally the granules may pass after some days through a filter plunged into sterile milk even without application of vacuum or pressure. In other words the granules are not simply filterable but actually migrant.

In conclusion, mention should be made of a stock culture of acido-proteolytic *Mammococcus* existing in two states, unfilterable and migrant-filterable, which differ not only morphologically but also physiologically. The filterable state is less active both chemically and culturally; it is unable to coagulate milk and is difficult to reproduce in ordinary mediums, but it may be readily cultivated in milk.

Consequently milk which I have found to be active in promoting bacterial physiologic dissociation (1921), may be considered advantageous also for morphologic dissociation and even for the growth of filterable and migrant forms. This is presumably related to its inclusion of native protein, which is particularly suitable to parasitic or semiparasitic germs like *Mammococcus*.

G12. *Filtrable Forms of the Rosenow Streptococcus from Anterior Poliomyelitis.* RUTH DEWEY RICHARDSON (introduced by R. R. Mellon), The Western Pennsylvania Hospital, Institute of Pathology, Pittsburgh.

An emulsion of the brains of rabbits succumbing to intracranial inoculations of the Rosenow poliomyelitis streptococcus was reinoculated into fresh rabbits. The brains of these were emulsified and filtered through Berkefeld N filters. The filtrates in doses of 0.5 cc. intracranially were innocuous despite the recovery of the original streptococcus from them by the Hauduroy serial transplant method on blood agar plates. The organisms appeared from the fourth to the sixth transplant.

Filtrates have also yielded positive results when the material filtered was recovered in fairly large amounts from the peritoneal cavities of rabbits inoculated with 20 cc. doses of the 24-hour brain-heart infusion broth culture of this streptococcus.

The same type of broth culture was filtered without being passed through rabbits as above. Prolonged plate cultivation was negative. The filtrates were then placed in sealed ampoules at room temperature and at 37.5°C. for five months. Plates made from those kept at 37.5°C. gave minute colonies, appearing at first as pleomorphic diphtheroids, but regained in full their original characteristics as green streptococci when grown for three days in whole blood. The ampoules kept at room temperature gave no growth on prolonged intensive cultivation.

The conclusion seems justifiable that under the conditions of the experiment the Rosenow anterior poliomyelitis streptococcus is filtrable; but this fact in itself by no means proves that the filtrable phase is identical with the virus of poliomyelitis. Monkey inoculations are in progress.

G13. *A Five-fold Technic for Producing the Filterable Form of Bacillus phytophthorus.* AGNES J. QUIRK, United States Department of Agriculture, Washington.

A five-fold technic is presented for causing the plant pathogen *Bacillus phytophthorus,* which produces a black rot in potato tubers to (1) dissociate into its pure smooth and rough forms at will, (2) become a filterable type of culture, (3) be placed in a state to pass the filter candle, and in a stage suitable for recovery, (4) be recovered from the filtrate by medium selection, and (5) be identified as the "normal" *Bacillus phytophthorus* organism by (*a*) characteristic growth in culture mediums, (*b*) virulence on raw potato tubers.

This paper is a presentation of facts as seen and recorded during the study of the pure S and R forms with no attempt to theorize or to interpret the various stages of the organism's development.

G14. Dissociation in the Spore-forming Aerobic Group. M. H. Soule, Hygienic Laboratory, University of Michigan, Ann Arbor.

Several strains of each of the following representatives of the "subtilis group" were selected: *B. mycoides, B. megatherium, B. mesentericus* and *B. cereus*, and their dissociation investigated. Strains from various American and European laboratories, as well as strains freshly isolated from air, milk and soil, were purified by single cell isolation and dissociated by growth in large volumes of broth and under the influence of homologous immune serums. The usual dissociants, designated S, R, M (mucoid) and P (phantom) according to the distinct character of growth on agar plates, were obtained, stabilized and studied. It is important to bear in mind that these forms do not necessarily correspond to the S and R variants obtained from old broth cultures of *B. dysentery, B. typhosus*, etc. by Arkwright. (Originally, colony appearance was the criterion for the designation S and R by this worker, however, he has recently discarded this character and now bases his designation on agglutinogenic content, stating very clearly, that it may be impossible to differentiate the variants by colony appearance. This change has resulted in, and will continue to cause considerable confusion among those working in the field of microbic dissociation.) The colonies described were obtained by streaking the inoculum in question on the surface of infusion agar. After incubation at 37°, the plates were examined and the colonies studied; thus the descriptions refer to colony types obtained under identical conditions.

The S variants, the type ordinarily encountered, formed shiny, round, soft, smooth, moist, compact colonies. The final stages, after continued incubation, frequently presented a variety of pictures.

The R variants formed large, flatly convex colonies with a rough surface, dull frosted appearance and an irregular border. On continued incubation the type of growth varied considerably.

The P or phantom type seems to be an intermediate form between the S and R, and appeared most frequently in cultures undergoing rapid dissociation, and for some strains it has not as yet been observed. The colonies varied a great deal in size; they were colorless, flat and visible only by reflected light or with the low power lens. When first isolated this type was usually labile with a decided tendency for the production of the R type. By the selection of colonies and suspending the inoculum in salt solution previous to the streaking of agar plates the P forms were stabilized. The individual cells in the colonies varied from granular material to well formed, actively motile rods. Growth in broth was extremely slow.

The M or mucoid type likewise appears to be an intermediate form between the S and R. The colonies were usually between the S and R types in size and were round and dome-shaped with a shiny, moist, mucoid appearance. On continued incubation the expected increase in size occurred, with a smooth, abrupt edge, unless the colonies were crowded too close together. On subculturing the typical M colonies ordinarily resulted. The individual cells were usually surrounded by very thick capsules. The growth of this type in broth was usually agglutinative with a marked tendency toward pellicle formation.

It is emphasized that many exceptions to the above descriptions were noted, but in general, colonies having the characteristics mentioned were obtained from each strain. Dissociation took place only in growing cultures. Spores and quiescent cells suspended in normal saline or in distilled water did not dissociate. The presence or absence of motility was not necessarily associated with colony type. Lack of motility, when noted, in any dissociant of a strain was usually found in the mucoid and R forms.

The R type seemed to be the most stable but dissociated readily when grown in broth containing homologous immune serum giving S, M and P forms. The dissociation of the S form toward the mucoid type was greater when incubation was carried out at 45° and 50°.

A limited growth of all types occurred in the absence of free O_2. The respiratory quotients for all strains and their dissociants were practically identical and on plain agar had an average value of 0.891. There was little difference in the production of indole and in the reduction of NO_3^- to NO_2^- and NH_3 by the variants of the same strain.

The S type was usually more strongly antigenic but the distinct S, O and R antigenic structures associated with colony type, as described by the English workers for the members of the enteric group, were not observed.

All types were non-virulent for guinea pigs and rabbits. A special effort to produce a filterable virus (encephalitic virus) from these strains resulted negatively. A good active lytic principle for these strains has not been obtained in the present investigation.

G15. Dissociation of Tubercle Bacilli, Particularly B.C.G. G. B. Reed, Queen's University, Kingston, Ont.

Forty cultures of tubercle bacilli including human, bovine and avian forms from widely differing sources have been compared. Recently isolated virulent cultures and cultures with a long history of high virulence all grew on gentian violet egg in S colonies; on fluid media, in the case of human and bovine types, as a continuous veil-like pellicle, in avian types as a diffuse suspension. All were agglutinated in buffer solutions of only high acidity. All the avirulent or low virulent cultures or those with a history of loss of virulence grew in R colonies on solid media, as a heavy pellicle tending to separate into discrete islands on fluids; and they agglutinated at relatively low acidity. The two types were also distinguished by their antigenic content as shown by complement fixation and specific agglutination.

Four cultures of B.C.G. apparently avirulent and R in colony form when received, have been cultured for one to three years in basic fluid media, mostly that of Proskeur and Beck. Originally all grew as a thick folded pellicle with a tendency to separate into island-like masses. After a few to many generations all the strains began to show minute patches of finely granular pellicle. The addition to the synthetic fluid of normal or anti-R rabbit serum or emulsions of heat killed R organisms appeared to somewhat promote this development. Repeated subculturing from the fragments of veil to fresh fluids resulted in practically pure veil-like growths. Plates made from the veil fragments on gentian violet egg developed definite S colonies in sharp contrast with the original R form. Both the veil and the S colonies produce in guinea pigs generalized, progressive tuberculosis and death in short periods. Tuberculous tissues of advanced cases or dead animals frequently show very large numbers of bacilli; cultures have repeatedly been obtained, and guinea pigs injected with exudates or macerated tissues have developed progressive, fatal disease. The S forms resemble other virulent S types.

The B.C.G., from the four strains examined, appears to be a highly stable R form of the tubercle bacillus which may, however, under appropriate cultural procedure continued over a sufficiently long period, dissociate into virulent S forms.

G16. Studies on the Dissociation of the Brucella Group. W. L. MALLMANN AND FRANK GALLO, Michigan State College, East Lansing.

Further studies on the relationship of the three species of Brucella, namely *Br. abortus*, *Br. suis* and *Br. melitensis* are needed, particularly studies on the dissociate forms of these organisms. Smooth strains of these organisms were dissociated in 10 per cent immune serum broth, 1 per cent lithium chloride broth, and by aging on liver agar slants. The latter method was the most successful. The rough strains were studied serologically, biochemically, culturally and morphologically to determine their relationship to the smooth strains. All of the rough strains on thionin and basic fuchsin agar plates behaved as *Br. melitensis.* The rough strains of *Br. abortus* failed to produce hydrogen sulphide while strains of *Br. melitensis* and *Br. suis* showed no change from the smooth types. Antigens prepared from the rough *Br. abortus* failed to agglutinate rough *Br. melitensis* and *Br. suis* serums. Rough *Br. melitensis* and *Br. suis* were closely related serologically. All rough strains reverted to their smooth prototype by animal passage.

G17. Dissociation of Staphylococcus aureus and Its Relation to Infection and Immunity. RACHEL E. HOFFSTADT AND GUY P. YOUMANS, University of Washington, Seattle.

Dissociation of a virulent strain of *Staphylococcus aureus* was undertaken by two methods: (1) Daily transfers in lithium chloride medium; (2) animal inoculations. Studies of the biochemical characteristics and serological reactions were made as well as of virulence of the strains dissociated.

Eight rough types and one "G," or filter-passing, strain were isolated. Four biochemical types were found differing not alone in the kind of carbohydrate fermented, but in the rate of fermentation. The "G" form had a higher thermal death point than either the rough or smooth types. Cross agglutinins and complement fixations were found for rough, smooth and "G" forms. No type was virulent except the original smooth yellow form. The "G" type reverted to a smooth yellow avirulent form with biochemical characteristics similar to the original form both in vitro and in vivo. This form was found as long as four weeks after the original inoculation of "G" into a rabbit in its heart's blood.

The "G" form was dissociated more rapidly in the animal than in lithium chloride medium. Previous treatment with heat-killed antigens of smooth, rough and "G" forms and the presence of specific agglutinins and complement fixations had no effect on the type or rate of dissociation. An animal which dissociated the smooth form either survived a lethal dose of the smooth yellow form or lived longer than one which did not dissociate it. From the above study it is felt that the dissociated forms represent cyclo-stages in the life history of *Staphylococcus aureus* rather than mutants.

G18. The Asparagin and Glycerin Metabolism of the Tubercle Bacillus.

John H. Dingle and John Weinzirl, The Alice McDermott Foundation, University of Washington, Seattle.

To determine the manner of utilization of asparagin and glycerin by tubercle bacilli, two strains of the organisms were grown on a special synthetic medium containing asparagin as the sole source of nitrogen and glycerin as the chief source of carbon. The two strains employed were a virulent H 37 and an avirulent H 520. The following analyses were made weekly upon the media: hydrogen-ion concentration, titratable reaction, ammonia, amino-nitrogen, total nitrogen, protein-nitrogen, rest nitrogen, and glycerin content.

The results obtained show that the virulent and avirulent organisms seem to be alike in their metabolism on the synthetic medium employed, except that the avirulent bacilli grew more rapidly and produced a terminal alkalinity, while the virulent H 37 grew more slowly and produced a terminal acidity. The titratable reaction and pH curves closely paralleled each other throughout the course of the experiment. Apparently there was no relationship between the titratable alkalinity and the ammonia content, for the latter was small, and showed a maximum content at the time of greatest growth followed by a recession.

The nitrogen determinations showed that practically all of the nitrogen of the asparagin was utilized by both organisms, the imino-nitrogen utilization taking place more readily than that of the amino-nitrogen. Both organisms utilized almost completely the glycerin in the medium; and it appeared that the glycerin was not partially oxidized to organic acids.

The acidity produced in the medium by the organisms seemed to a considerable extent to be due to the metabolism of the asparagin. The terminal alkalinity produced by the avirulent organisms is explained by the assumption that there occurred further utilization of the acidic compounds produced from the asparagin.

G19. *A Note on the Use of Crystal Violet in Presumptive Tests for Water Pollution.* C. N. STARK AND C. W. ENGLAND, Cornell University, Ithaca, N. Y.

During recent years many attempts have been made to devise a medium which will eliminate false positive presumptive tests in water analysis. The demand for a medium which will give more clear cut results has increased. Conversely, the proper safeguarding of our water supplies demands that a medium be used which will give positive results on all polluted waters.

Certain dyes have been added to the test medium with the hope of eliminating positive presumptive tests of doubtful significance. Salle (Jour. Bact., 1930) has recommended a crystal violet broth for this purpose. His medium has been tested by us on colon organisms recently isolated from human feces. The crystal violet in this broth has been found to have an inhibiting action on fecal organisms. Of ten persons, from whose feces colon organisms were tested, four were found to contain organisms which gave negative results after 48 hours incubation in Salle's crystal violet broth. Thirty-two of 91 fecal cultures tested by us gave negative results in this broth. All of these cultures gave positive results in standard lactose broth and also in Salle's medium when the cystal violet was omitted.

Our results with Salle's medium show that it should not be employed as a standard medium for water analysis and that a medium which will give positive results on all polluted waters should be used.

G20. *Types of Bacteria on Blood and Chocolate Agar and the Immediate Cause of These Types.* EINAR LEIFSON, Johns Hopkins Medical School, Baltimore.

From a study of certain Gram-negative bacilli and 15 selected strains of streptococci and pneumococci 5 distinct types were differentiated as to their action on blood and chocolate agar. The appearance of the deep colonies of these types as well as the probable cause of such appearance is given in the following table:

Type	*Blood agar*	*Chocolate agar*
Gamma	No visible effect	No visible effect
Delta	Large green zone with little if any hemolysis. Appearance due to H_2S production and subsequent oxidation by air of the sulfhemoglobin formed	No visible effect
Alpha	Green zones with partial hemolysis. Appearance due to the formation of hematin, and the interaction of hematin and H_2O_2. Hemolysis probably due to hemolytic action of H_2O_2	Green coloration. Due to interaction of hematin and H_2O_2
Beta	Large zones of complete hemolysis. No green	No visible effect
Beta prime	Large zones of complete hemolysis. No green	Green coloration. Due to interaction of hematin and H_2O_2

Nothing definite was discovered about the cause of the beta type of hemolysis. The delta type is produced by most green producing bacilli and the alpha type by most green producing cocci. The production of a green coloration by the delta type seems always to be correlated with production of H_2S. Addition of H_2S to blood first produces a purple coloration of sulfhemoglobin which upon exposure to oxygen turns green. If a blood plate is exposed to H_2S it turns green immediately. Under anaerobic conditions H_2S does not turn blood green, and delta bacteria do not produce a green coloration in blood agar. Hydrogen sulphide has no visible effect on chocolate agar and neither do delta bacteria.

The cause of the alpha type has been recognized more or less for some time. All alpha bacteria produce a green coloration of chocolate agar which shows that they produce hydrogen peroxide. The green coloration most likely results from the interaction of H_2O_2 and hematin which is formed when hemoglobin is heated. Hydrogen peroxide does not react with hemoglobin or methemoglobin to form a green compound. Most alpha bacteria produce methemoglobin to a greater or less extent. It is probable, therefore, that the hemoglobin is changed first to methemoglobin and this in turn is changed to hematin. The hematin then reacts with H_2O_2 to form the green compound. Under anaerobic conditions alpha bacteria produce neither methemoglobin nor H_2O_2, and in blood agar under the same conditions produce no green coloration and no hemolysis. Since the hemolysis by the alpha type appears only under conditions which also seem to lead to the production of H_2O_2, and since H_2O_2 is fairly hemolytic, it is most likely the cause of the hemolysis.

The nature of the medium and the conditions of incubation are important in producing definite and consistent results. Bacteria, otherwise delta, generally fail to produce a green coloration on synthetic media. On media containing cysteine or sodium thiosulphate gamma bacteria may produce a green coloration. On synthetic chocolate agar, alpha bacteria generally fail to produce a green coloration. Typhoid bacilli often fail to produce any green coloration on extract blood agar while they do so on infusion blood agar. Under anaerobic conditions a green coloration is seldom produced. In some cases more green coloration is produced at 32°C. than at 37°C. These considerations should lead to a standardization of technique. It is especially to be emphasized that deep colonies and surface colonies do not always have the same appearance. Bacteria producing deep colonies of the gamma type often produce surface colonies of the hemolytic type. This paper is based altogether on the study of deep colonies.

G21. *The Appearances Produced in Blood Agar by Bacteria of the Bacterium coli Group.* Moses Paulson and J. Howard Brown, Johns Hopkins Hospital and University, Baltimore.

In blood agar plates bacilli of the *Bact. coli* group produce appearances similar to those of the alpha, alpha prime, beta, gamma and delta types of streptococci. Gamma types were found to be three times as numerous as beta; the alpha type was represented by less than thirteen per cent of the strains studied. In the human rectums cultured there was no obvious relation of the blood agar types of bacilli isolated to the clinical conditions. The various types were isolated with about equal frequency from normal bowels and those affected with organic intestinal disease (chronic ulcerative colitis). Such, however, may not be the case with known *Bact. coli* infections. As with the streptococci, the importance of studying the deep colonies under the microscope and the importance of employing a medium of uniform composition and uniform conditions of incubation and observation must be emphasized. Similar results were obtained with human as with rabbit blood, such differences as were noted being quantitative rather than qualitative. The determination of the type of appearance produced in blood agar should be a useful adjunct in the study of bacilli of the *Bact. coli* group.

G22. Vegetable Peptone Agar for Quantitative Work with Lactobacillus acidophilus. FREDA M. BACHMANN AND W. D. FROST, The Stout Institute, Menomonie, Wis., and University of Wisconsin, Madison.

The use of a vegetable peptone agar is suggested for quantitative determinations of *L. acidophilus* products. The organism was found to grow well in decoctions from various vegetables, but especially well when cabbage, carrots, tomatoes, or green beans were used.

Comparative data on quantitative determinations with dehydrated galactose whey agar and with a cabbage peptone agar, indicate that the latter medium is to be preferred. The claims made for it are: first, it is easily prepared since the ingredients are readily obtainable, and adjustment of reaction is not necessary; second, the medium is very clear and the colonies easily seen, and small colonies are not likely to be confused with detritus; third, the colonies usually grow to good size; fourth, it encourages the characteristic rough or X type of colony; fifth, the counts are usually slightly higher than on the galactose whey agar plates, especially with the older milks.

G23. A. Taxonomic Study of the Acidophilus Group. H. R. CURRAN AND L. A. ROGERS, Bureau of Dairy Industry, Washington, D. C.

The relationships of about 100 acidophilus cultures including dental and Doederlein strains have been studied. Groupings are made on the basis of volatile acid production, tolerance of indole and phenol, temperature relationships, sugar fermentations, limiting undissociated lactic acid and other physiological reactions.

G24. A Cultural Study of the Propionic-acid Bacteria. E. R. Hitchner, Department of Agricultural Bacteriology, University of Wisconsin, Madison.

In the present investigation a cultural and physiological study has been made of 22 cultures of the propionic-acid bacteria selected from 43 cultures isolated from Emmenthal cheese, Edam cheese, milk, soil, and silage together with 19 cultures obtained from other workers. A procedure found quite satisfactory for the isolation of these microörganisms consists of preliminary enrichment in sodium lactate yeast-water followed by streaking on dextrose yeast-water agar plates. Anaerobiosis is insured through the use of the anaerobic plate method described by Snieszko (Centbl. Bakt., Abt. II, **82**, 109, 1930).

The cultures exhibit marked variations in morphology, especially when they are grown aerobically or in a medium which becomes acid during their development. Many cultures produce pigments when grown in a medium in which a neutral reaction is maintained during growth. The development of the propionic-acid bacteria is stimulated by associated growth with other organisms. Data are submitted which indicate that this stimulatory effect is due in part to the production of more favorable growth conditions occasioned by a change in the oxidation-reduction potential of the medium.

Striking variations have been found in the catalase content of the cultures. Quantitative measurements of the hydrogen peroxide decomposed in a definite period showed that while most of the organisms cause a rapid destruction of this substance 5 cultures destroy only minute quantities. From these observations it would appear that the general characterization of the propionic-acid bacteria as vigorous producers of catalase should be modified.

The fermentative properties of the cultures have been tested in 21 carbohydrates, polyalcohols, and glucosides. Marked differences are found among the organisms both in their ability to utilize these compounds and in the amounts of acid produced. The hexose sugars and glycerol are fermented by all cultures; dulcitol and inulin are not fermented. From a diagnostic standpoint the most valuable information is furnished by 8 test substances, namely, xylose, arabinose, lactose, sucrose, raffinose, glycerol, mannitol, and starch.

Propionic acid, acetic acid, and carbon dioxide are the chief products formed by the propionic-acid bacteria in the fermentation of glucose. Small amounts of non-volatile acids, chiefly succinic acid, are produced. When the organisms are grown in a yeast-water medium containing approximately 2 per cent of glucose, the ratio of propionic acid to acetic acid varies from 1.8:1 to 3.9:1 with the different cultures. In general those organisms which ferment the largest number of test substances produce the highest ratio of propionic acid to acetic acid. Succinic acid is not utilized by any of the cultures when it is present as the sole source of carbon. However, data are submitted which show that with some strains volatile acids may be produced from succinates in the presence of a fermentable carbohydrate such as glucose.

All cultures exhibit the general characteristics ascribed to the genus *Propionibacterium* (Orla-Jensen, 1909). On the basis of morphology in acid media, chromogenesis, action on milk, nitrate reduction and fermentative properties, these cultures have been separated into 8 groups, 6 of which are similar to or identical with certain species recognized by Van Niel. The other groups deviate sufficiently in their characteristics from previously described types to warrant the creating of two new species, which have been designated *Propionibacterium zeae* n. sp. and *Propionibacterium arabinosum* n. sp.

G25. A New Bacterial Species Isolated from Strawberries. HELEN F. SMART, United States Department of Agriculture, Washington, D. C.

In the course of a microbiological investigation of strawberries, the occurrence of the same species of bacterium in great numbers in many samples of fresh fruit was noted in 1929 and 1930. This species was the predominating one in samples of strawberries from four commercial packing plants in Virginia, Maryland, and Delaware. It is described as a non-motile, gram-negative, non-spore-forming rod with an average size of 1.5μ by 0.75μ. Agar colonies are circular, smooth, glistening, bluish white. Gelatin is not liquefied. Growth is grayish white on potato, with weak diastatic action. Optimum temperature 26° to 31°C. Does not form indole, H_2S or ammonia. Nitrates are reduced. Produces acid curd and deep brown color in milk; reduces litmus in milk. Forms acid and no gas in glucose, lactose, dextrose, glycerol and mannitol; gives an alkaline reaction and no gas in sucrose. The name *Achromobacter delmarvae* is proposed.

G26. Bacteria in Ocean Waters and Sea Bottom and Their Rôle in the Cycle of Elements in the Sea. SELMAN A. WAKSMAN AND H. W. REUSZER, Department of Marine Bacteriology, Woods Hole Oceanographic Institution, and Department of Soil Microbiology, New Jersey Agricultural Experiment Station.

The numbers of bacteria found in sea water and in the surface layers of the sea bottom depend entirely upon the distance of the area from land, upon the depth of the sample, upon the region, and upon a number of other factors. The plate method is commonly used for this purpose, although it is known that only a small part of the bacteria found in sea water are able to develop on the plate. The following numbers of bacteria were found in the neighborhood of Woods Hole, Mass.:

SOURCE	DEPTH FROM SURFACE OF WATER	BACTERIA IN 1 CC.	
		Aerobic	Anaerobic
Samples obtained from Massachusetts Bay			
	meters		
Sea water	3	55,000–326,000	7,000–40,000
	55	466,000	40,000
Bottom mud	56–91	14,000–430,000	3,300–40,000
Samples obtained off the coast of Martha's Vineyard			
Sea water	Surface	27–735	
	Bottom (18–570)	159–90,700	
Bottom mud	—	200– 4,200	

Among the specific bacteria, the presence of aerobic and anaerobic cellulose-decomposing, agar-liquefying, nitrogen-fixing organisms, and others has been demonstrated. Some of these bacteria have been isolated and are being studied further.

G27. *A Coagulable Sugar-Free Base for Fermentation Studies.* ROBB SPALDING SPRAY, School of Medicine, West Virginia University, Morgantown.

A simple basic medium may be prepared from 1 per cent nutrose (sodium caseinate), 1 per cent peptone (Difco) and 0.5 per cent powdered egg albumin (Difco), dissolved in 100 ml. of distilled water, adding suitable indicator. These substances dissolve readily and leave a fluid medium after autoclaving. In the above proportions the final reaction is usually pH 7.0 without adjustment. Fermentation tests and tests for reducing sugars show the medium to be sugar-free. Any desired carbohydrate may be added, preferably as an unheated, filtered solution, which necessitates aseptic tubing and the usual incubation for sterility. Carbohydrates not affected by heat may be added previous to autoclaving.

This medium supports vigorous growth of the colon-typhoid group, staphylococci and streptococci and many others. Fermentation results in coagulation within 8 to 12 hours, and formation of gas is readily revealed by the entrained bubbles.

The medium is an effective substitute for the Hiss serum-water, and has the advantage of requiring no manipulation to render sugar-free.

G28. *A Comparison of Different Bacteriological Counting Methods.* BERNHARD DOMOGALLA, City of Madison and Wisconsin State Laboratory of Hygiene.

A study of the bacteriological count of raw lake waters, drinking waters, river waters, as well as sewage waters was made using different culture media and the new modified, rapid, direct counting method. In all cases the total number of bacteria by this modified direct counting method was from 100 to 1000 higher than the total number of bacteria found growing on any culture medium. The results as well as a sketch of the equipment used in preparing the water samples for the direct count will be shown by lantern slides.

G29. The Preservation of Bacteria in Vacuo, II. J. HOWARD BROWN. Johns Hopkins University, Baltimore.

In January, 1925, a first report on the preservation of bacteria in vacuo was made to the Society. These experiments were started in 1917 at which time only 3 strains of pneumococci (types I, II and III) were dried on bits of glass cover slips over calcium chloride in a desiccator from which the air was partially evacuated. These cultures remained viable for less than 4 years. In 1919 five strains of beta hemolytic streptococci were similarly dried and placed in the desiccator. In 1923 the dried streptococci were transferred to specially ground milk bottles and cultures of some other organisms were similarly preserved. A higher vacuum was established. Since then perhaps 200 or 300 cultures have been preserved in this manner.

Recently some of the oldest bottles have been opened and the dried preparations cultured. The 5 streptococci dried in 1919 are all viable (12 years). A type II pneumococcus dried in 1919 in the poorly evacuated desiccator was viable for not quite 4 years but 5 strains of pneumococci I, II and III dried and preserved in the more highly evacuated milk bottles since 1923 are all viable (8 years). Of 17 strains of streptococci and 5 of *Corynebacterium pyogenes* dried in 1923 all but one streptococcus are viable. One strain of *C. diphtheriae* dried in 1924 is viable and toxicogenic after 7 years. One strain of *Bact. bronchisepticus* dried in 1925, four strains of scarlet fever streptococci dried in 1926 and 1 guinea pig pneumococcus dried in 1927 are viable. At the present writing, of 40 strains of bacteria dried in vacuo from 4 to 12 years ago all but 2 have remained viable.

As the technique has been simplified and improved better results have been obtained. The technique employed at present will be demonstrated.

G30. *The Process of Spore Formation in B. megatherium as Shown by Motion Pictures.*

STANHOPE BAYNE-JONES AND ALEXANDER PETRILLI, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, N. Y.

The object of the investigation was to study the process of spore formation in *B. megatherium* by means of a continuous photographic record on 16 mm. motion picture film.

The organisms were grown at 37.5°C. on small cones of 2 per cent agar containing 0.05 per cent peptone or under thin hanging drops of the usual 1.5 to 2 per cent peptone agar in special culture chambers adapted to the growth requirements of the organism and the optical system of the apparatus. Photographs of cells under observation were taken at rates of 7.5 to 30 per minute. A study of the process of spore formation was made from projections of these films.

During the first phases of rapid multiplication, the cells were almost devoid of granules. These granules increased in size and number after about the fourth to sixth hour depending upon the type of medium used. Growth stopped at about the eighth to tenth hour and granules disappeared from the ends of many of the cells. Numerous large and small granules remained in the ends opposite the cleared area. These showed active brownian movement but never penetrated the cleared ends. After 12 to 14 hours, spore formation occurred regularly in the cleared ends of the cells. The oval spores appeared suddenly, within 5 to 20 minutes in the ends of the cells which during the preceding two hours or more had been hyaline. The spores were not formed from any observable antecedent granules. Apparently, the region in the cell in which the spore is to be formed undergoes some sort of condensation, free from visible granules, several hours before final completion of the spore, or is separated by a membrane from the granular region of the cell. Inadequate supply of oxygen, drying and the supply of excess of peptone with respect to the supply of oxygen inhibited spore formation. It seemed apparent that spore formation required certain optimum conditions for growth and did not arise in response to an unfavorable environment.

G31. *The Nature of the Infection Thread in Leguminous Root Nodules.* ELIZABETH McCOY, University of Wisconsin, Madison.

From the time of Eriksson, 1873, to the present day there have been two opposing views on the nature of the infection thread in leguminous root nodules: namely, that the thread is a zoogleal strand imbedding the bacteria or that it is a definite tube in which the bacteria lie imbedded in their own slime.

In the course of a microchemical study of nodule tissues some observations were made which seem to support the second of these conceptions of the infection thread. With a Chambers micromanipulator pieces of infection thread may be easily dissected out and shown to be relatively rigid structures. When cut or crushed with the needles they show ragged edges like broken plant cell walls. Furthermore the isolated pieces of infection thread are obviously insoluble in water (as they should be if consisting only of gum elaborated by the rhizobia). Such physical properties strongly suggest the presence of a rigid outer layer, or sheath.

A microchemical study was therefore made in order to discover the composition of the sheath and its probable origin—from plant or bacteria. On the basis of solubility and staining reactions the following constituents are believed to be present: cellulose, hemicellulose, and perhaps pectic material (not calcium pectate). In the course of the cellulose tests involving separation of the cells owing to the previous removal of the binding material, it was demonstrated that the *sheaths of the infection threads do not extend across the middle lamellae.* In other words, they are a deposit of the individual plant cells, and from the fact that cells are sometimes found in which the sheath appears only close to the cell wall, it is suggested that the sheath is formed by differentiation inward from the cell wall, not by deposit from the protoplasm along the original strand of infection.

G32. *Experimental Comparison of the Different Definitions of Death.* OTTO RAHN AND MARGARET N. BARNES, Cornell University, Ithaca, N. Y.

Death is defined in many different ways, and always by the loss of some property characteristic to the living organism. Compelled by the technique, bacteriologists usually define a cell as dead when it has lost permanently the power of reproduction. This definition is used almost exclusively by bacteriologists while other biologists usually consider a cell dead when it has lost the power of respiration (or fermentation) or the power of reduction (methylene blue) or the power of selective permeability as evidenced by loss of plasmolysis or by adsorption of dyes.

While some authors have assumed these different definitions to indicate the same degree of damage done to the cell, others have believed them to indicate different stages of a chemical change taking place in the cell under adverse conditions.

Experiments were carried out with yeast exposed to heat, ultraviolet light and chemical poison. The amount of "death" according to the different definitions was measured. There was considerable difference in the time required to bring about the various symptoms of death under uniform conditions. In all experiments, loss of reproduction occurred earlier than any of the other symptoms. The order of the other symptoms varied a little with different causes of death.

The loss of reproductive power is not necessarily followed by an early loss of fermenting power, reducing power or selective permeability.

G33. Does Freezing Kill Cl. botulinum Spores? LAWRENCE H. JAMES, Bureau of Chemistry and Soils, Washington.

The production and marketing of frozen vegetables and fruits without heat sterilization has raised the question as to whether or not there is a possibility of botulinus poisoning arising from the consumption of such products which had been improperly handled.

Two general methods are used in the freezing of food products, "quick" freezing in which the food material is solidified in a comparatively short time by exposure to very low temperatures, ranging from −50° to −85°F., and "slow" freezing in which the packaged materials are placed in a refrigerated room and solidified only after several hours of exposure. Freezing the spores with solid carbon dioxide ice resembles the treatment in "quick" freezing, and exposure in a cold room is comparable with "slow" freezing. The effect of freezing upon the botulinus spores is obviously of first importance, and studies have been made in which the conditions of experimentation have resembled those existing in commercial practice. Dried *Cl. botulinum* spores (Type B) suspended and heated in a Sörensen buffer solution of pH 6.9 have been frozen with solid carbon dioxide ice, defrosted at intervals, and the total numbers of living spores as well as the presence of toxin determined. There was no reduction in the number of living spores, neither when defrosted and examined immediately after being frozen, nor when they have been frozen for 9 days and then examined. Subcutaneous injections into guinea pigs showed that no toxin had been liberated from the spores by the freezing.

A suspension of dried spores of the same botulinus culture was divided among several test tubes and frozen by exposure in a refrigerated room held at approximately 20°F. Twenty-four to thirty-six hours were required for the suspensions to be thoroughly solidified. Examination of portions one day after being frozen and periodically to eleven weeks showed that there was neither a reduction in the total numbers of living spores, nor was toxin liberated.

G34. Paramecia as a Test of Bacterial Inhibition. JEAN BROADHURST AND JEAN ORAM, Teachers College, Columbia University, New York.

The possible use of paramecia, or other living cells, as a ready index of the germicidal or inhibitive values of specific chemical preparations has been studied by repeated tests on "standardized" paramecium cultures during a period of more than 2 years with over 40 common or widely advertised disinfectants and antiseptics.

The paramecia were cultivated in autoclaved hay infusion which was later inoculated with two kinds of bacteria: *Flavo-*

bacterium trifolium (sp.?) and *Alkaligenes fecalis.* Four to six day cultures yielded the most reliable results, the paramecia showing the greatest resistance at that age, and the results of duplicate tests being more consistent than with younger or older cultures.

The tests were made with concave slides, mixing 0.2 cc. of a four-day paramecium culture (usually 15 to 20 paramecia) with 0.4 cc. of the chemical to be tested; the effects were observed with the low powers of the compound microscope and recorded in terms of visible changes in the cell and the time interval preceding death. Inhalants or volatile substances were tested by inverting a concave slide containing one drop of the paramecium culture over another concave slide containing 0.4 cc. of the chemical preparation, the hollow of the lower slide having been previously ringed with vaseline to act as a seal.

Various types of cell effects were observed, mainly extreme agitation, distortion, formation of bubbles or "blisters," the marked extension of the trichocysts, the rupturing of the cell, and "fixity" or death not necessarily accompanied by any of the other effects.

Being primarily interested in the use of paramecia as a measure of the value of mouth washes and similar preparations, we made parallel tests on the inhibitive action of the preparation on *Staphylococcus aureus,* using the filter paper agar plate method (The American Journal of Nursing, November, 1931).

The comparative results obtained with paramecia and *Staphylococcus aureus* will be shown in charts. It is sufficient here to state what our results show no uniform correlation between the inhibiting value of chemicals on *Staphylococcus aureus* and the effect on paramecia, either with regard to actual destruction of the paramecia or any of the various visible effects listed. It is also interesting to note that the effect on paramecia varied greatly within a given class of substances, e.g., one organic acid causes distortion and extension of the trichocysts before death, while another causes death without any change in the cell form. Our results also indicate that it is not possible to "scale" or relate the different visible effects to the varying concentrations of a given preparation, no correlation being found between such effects as bubbles or distortion and the dilutions used. It is possible that having previously determined the type of effect produced by a specific dilution of a given chemical, standardized paramecia might later be used to check on unknown dilutions of that substance. In most cases, however, it would be misleading to interpret death, or any change in cell structure, as indicating possible bactericidal value in the preparation. For example, our results show that physiological salt solution causes distortion and bursting with death in two minutes, and some of the most inert or useless preparations from an inhibitive or germicidal point of view cause much more violent effects on paramecia than proven germicidal substances.

G35. The Relation of Temperature to the Germicidal Efficiency of Lye Solutions. ERNEST C. MCCULLOCH, University of Wisconsin, Madison.

Experiments pursued during the last year show that dilute solutions of high-grade household lye are very efficient in destroying *Br. abortus*, *E. coli* and related microorganisms. Using the Reddish technic, a 1:800 solution destroyed cultures of *E. coli* within a few minutes and lye was found to have a phenol coefficient of approximately eight. In studies in barn disinfection, no non-sporulating organisms were recovered in subculture from floors or gutters after five minutes exposure to a 1:150 lye solution.

A decrease in temperature was found to exert relatively small influence upon the germicidal efficiency of lye solutions. Little consistent difference in the killing time was noted in trials made at 2° and 25°C. Electrometric pH determinations of the lye solutions at various temperatures indicate that the ratio of hydroxyl-ion to hydrogen-ion activity decreases with increase in temperature.

This might be accounted for by the assumption that with an increase in temperature the mobility of the hydrogen-ions is more markedly increased than is the mobility of the hydroxyl-ions. Heating would thus cause an increased ratio of hydrogen-ion to hydroxyl-ion activity and cooling would result in an increased ratio of hydroxyl-ion to hydrogen-ion activity.

The data suggest that the activity of the hydroxyl-ions is responsible for the destruction of bacteria when exposed to lye solutions. At temperatures between 2° and 25°C. the decreased ratio of hydroxyl-ion to hydrogen-ion activity approximately balances the increased germicidal effect of each individual hydroxyl-ion at the higher temperatures, thus giving little difference in germicidal effect with temperature changes.

G36. The Effect of Cell Concentration and Age of Culture Upon the Mortality of Bacteria Exposed to Alkalinity and Heat.

J. H. Watkins and C.-E. A. Winslow, Department of Public Health, Yale School of Medicine, New Haven, Conn.

The mortality of vegetative cells of *Es. coli* in sodium hydroxide solutions appears to follow the usual rule of logarithmic decrease and does not show a progressively increasing rate as was found to be the case for spore formers by Levine and Meyer.

The slope of the mortality curve is very definitely correlated with the initial concentration of cells exposed to the action of the disinfectant,—larger initial numbers showing a slower decrease. The relation between K (the mortality constant) and the logarithm of initial concentration is essentially a linear one.

The same general relation between initial cell concentration and rate of mortality holds when the cells are heat-killed by exposure in distilled water to a temperature of 55°.

We can see no escape from the conclusion that the "safety in numbers" must lie in the production by the organism of some protective substance adjacent to the cell which is weakened by dilution. In the case of heat-killing the presence of such substances in the protective zone must presumably cause in its turn some condition of the cell membranes which enhances resistance.

Suspensions of cells from old cultures show a higher degree of resistance than those prepared from young cultures. It would seem possible that the cells from older cultures are better able to produce the protective substances which are operative.

G37. *The Effect of Temperature upon the Rate of Growth of E. coli in Broth.* M. W. JENNISON[1] AND J. W. M. BUNKER, Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge.

Two strains of typical *E. coli* were used, an old stock culture designated C_1 and a recently isolated strain C_2. Both were grown at 22°, 27°, 32°, 37°, and 42°C. on agar slants, with weekly transplants, for about four months; then in nutrient broth, with two daily transfers, for a week before use. Rates of growth were determined in broth, using an original inoculation of 1000 to 2000 cells per milliliter from a 12-hour culture. Two series of growth rates were determined with each strain: (*a*) the rate at each temperature of the organisms "acclimatized" to 22°, and (*b*) the rate at each temperature of the organisms "acclimatized" to that temperature. Counts were made at the start and every two hours, from five plates of appropriate dilutions of pooled duplicate tubes. The temperatures did not vary more than ±0.15°C. from those stated, and precautions were taken to keep all other factors as constant as possible. The five temperatures of each series were run at the same time.

Under the conditions of the experiments, and using the minimum generation time (G.T.) of the logarithmic growth phase at each temperature, practically no difference in G.T. was found in either strain between series *a* and series *b*, there apparently being no acclimatization effect. The G.T.'s varied from about 65 minutes at 22° to 20 minutes at 37°. The recently isolated strain, in both series, showed an increase in the G.T. above 37°, whereas the old strain had practically the same G.T. at 42° as at 37°.

The values of $Q_{10°}$ were of the order often found in reactions of this type, and were nearly the same with both strains and both series; for example, in strain 2, series *a*, 22°–32°, $Q_{10°} = 2.5$; 27°–37°, 2.0; 32°–42°, 1.0.

None of the growth curves, as shown by their points of inflection, were of the symmetrical type characteristic of autocatalytic reactions, although in every case the curve at the "optimum temperature" was more nearly so than the others.

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[1] Portion of a thesis submitted for the degree of Doctor of Philosophy in Bacteriology, Massachusetts Institute of Technology.

Plotting the logarithms of the velocity constant k at each temperature, against the reciprocal of the absolute temperature, it was found that in all cases log k varied continuously with the temperature, and the thermal increment μ of the van't Hoff-Arrhenius equation decreases continuously, though slowly, with the rise in temperature up to a "critical temperature," about 37° in both strains, where the change is very marked. With the recently isolated strain, however, the drop is greater above 37°.

G38. *Metabolic Activity of the Bacterial Cell at Various Phases of the Population Cycle.* HAROLD H. WALKER AND C.-E. A. WINSLOW, Department of Public Health, Yale School of Medicine, New Haven, Conn.

Bacteria of the colon group have been cultivated in vessels continuously aerated during the course of the experiment with air which had been freed from carbon dioxide and ammonia. The effect of the aeration procedure on growth was determined and average curves plotted for the population cycle. Specific growth phases of short duration were selected at various points on the population cycle and simultaneous determinations made, at the end of each phase, of bacterial increase, reaction change in the medium, sugar consumption, change in CO_2 and NH_3 content of the medium, and of aeratable CO_2 and NH_3 collected by the air train during the period.

This method of cultivating bacteria in a continuously aerated medium gives a more normal picture of the life cycle of a bacterial population than that obtained in ordinary techniques which involve the rapid inhibition of the organisms by their own waste products. Three basic media were used, one per cent peptone solution, a solution containing 0.5 per cent peptone and 0.5 per cent lactose and a simple synthetic medium (the Dolloff medium) containing ammonium tartrate as a source of nitrogen and lactose as a source of energy.

The process of aeration, it is interesting to note, is highly favorable to development in the lactose and peptone media through the removal of toxic waste products, but is distinctly inhibitory in the simple synthetic medium, presumably through the removal of growth stimulating substances.

The principal results to date appear to be the demonstration that the production of both CO_2 and ammonia-nitrogen per cell increases enormously during the period of active multiplication or logarithmic increase. Late in the preliminary lag period and in the phase of logarithmic increase the culture produced 40 to 100×10^{-11} mgm. of CO_2 per cell per hour, while in the later period of stability the figure fell to 1 to 2×10^{-11}. For NH_3-N, the rate of production during the late lag phase was about 15×10^{-11} mgm. per cell per hour and for the phase of logarithmic increase, 4×10^{-11} while for the phase of ultimate stability ammonia production fell to 0.2×10^{-11}.

During the lag phase the ratio of CO_2 to NH_3-N is about 4, during the logarithmic phase over 10 and during the period of stability when growth balances death about 6, expressed in relative weights of the gases yielded.

If we compare the metabolism of the cells during the period of lag and of logarithmic increase with their metabolism during stability of population we find that CO_2 production is increased thirty-fold and NH_3-N production 20 to 70 fold. The ammonia formed may of course be largely derived from extracellular decomposition of peptone. The increase of CO_2 production however would seem to be a measure of increased metabolism due to the physiological condition of the actively multiplying cells.

It is of interest to note that the presence of carbohydrate in the medium decreases ammonia production but does not increase CO_2 production. It would appear that the phenomenon of gas production as observed in the ordinary fermentation tube may be related to effects on the solubility of CO_2 rather than to variations in the actual production of this gas.

G39. *The Use of Reduced Iron in the Cultivation of Anaerobic Bacteria.* E. G. Hastings and Elizabeth McCoy, University of Wisconsin, Madison.

Inconsistent results obtained in the testing of milk for the spores of anaerobic bacteria are the rule rather than the exception. In an effort to lessen the irregularities reduced iron was added to the milk before heating. The comparative results obtained in a series of tests of commercial milks with and without iron indicated the value of the iron.

Forty samples of certified milk were examined, 4 of which gave positive results when the iron was used and 2 when the iron was not used.

Twenty-four students out of a class of 28 obtained evidence of the presence of the spores of anaerobic bacteria in the milks tested when iron was present, while only 7 of the group succeeded in demonstrating their presence in the same milks when iron was not present.

Clostridium amylobacter and *Clostridium sporogenes* were grown in milk containing reduced iron with no other means of insuring anaerobic conditions. The inoculum in each of the six serial transfers during a 14-day period was a loopful of the previous culture. A more extended test of the continued cultivation of *Clostridium acetobutylicum* was made, the period was 112 days during which 42 serial transfers were made. The reduced iron was added to the milk before sterilization, the inoculum was a loopful of the previous culture.

The first lot of the medium was used during a period of 28 days, the second during a period of 33 days, and the third during a period of 51 days. In each instance the medium was stored in plugged test tubes and was not reheated or otherwise treated before being inoculated.

The same culture in the milk without the iron gave 13 positive results and 12 negative. The longest period during which a serial culture was successful lasted 12 days with 6 serial transfers. Whenever the series made in the milk without iron was broken, recourse was had to the milk-iron series for inocula.

The addition of iron to corn mash assists in the isolation of anaerobes which ferment starch, and in the determination of the heat resistance of their spores. In the latter case the addition of iron, even 4 days after the beginning of incubation served to initiate growth in the previously dormant spore tubes.

The iron seems to exert its stimulating action through the establishment of a favorable oxidation-reduction environment.

G40. The Influence of the Oxidation-reduction Character of the Medium on the Aerobic Growth of Certain Bacteria.

L. W. BROWN AND I. L. BALDWIN, University of Wisconsin, Madison.

The oxidation-reduction character of the medium has been shown to influence the reactions of several common aerobic and facultative bacteria toward atmospheric oxygen, as well as to determine, to some extent, the ability of small inocula to initiate growth.

The addition of 0.01 to 0.05 per cent $KMnO_4$ or 1:1000 H_2O_2 to agar shake cultures in various media (veal infusion, mannitol-nitrate, yeast-water glucose, nitrate, etc.) inhibited or entirely prevented the normal heavy surface growth of *B. subtilis*, *B. mycoides*, *Serr. marcescens*, *E. coli*, *B. graveolens*, *B. radiobacter*, and others, but instead served to induce a diffused growth throughout the agar, or in some cases a definite zone of growth at depths of 2 to 20 mm. below the surface.

Although small inocula of these organisms (10^{-6} or 10^{-8} dilutions of 24 to 48-hour cultures) were unable to initiate growth in various liquid media (beef-extract peptone, mannitol-nitrate, yeast-water glucose, yeast-water sucrose, etc.) within a week, the addition of small amounts of a reducing agent (0.005 to 0.02 per cent of thioglycollic acid depending upon the medium) permitted good growth with these inocula in 24 to 48 hours, and often increased the rate of growth obtained from larger inocula.

The pigment produced by *Serr. marcescens* on various agar media has been intensified by the addition of peroxide, and diminished by reducing the media with thioglycollic acid.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

A1. A Study of Escherichia coli in Ice Cream.

J. VENCESLAV ANZULOVIC, Department of Bacteriology and Pathology, University of Maryland, College Park.

This study was undertaken primarily to see if *E. coli* would survive pasteurizing temperatures in ice cream mix. Seventy-three samples of ice cream were examined and it was found that they contained from 200 to 157,000 bacteria per cubic centimeter. Forty-five samples produced gas and 21 produced a metallic sheen on eosin-methylene-blue agar. The majority of the samples were slow acid-producers in litmus milk.

A gram-positive organism was found that produced a metallic sheen on eosin-methylene-blue agar. When incubated in gentian-violet-lactose-peptone bile in combination with *Aerobacter aerogenes* and streaked on eosin-methylene-blue agar, it gave the partially confirmed tests for *E. coli.* This organism is still being studied.

Two *E. coli* cultures survived 62.8°C. in milk; 13 cultures survived 62.8°C. in ice cream mix. At 65.5°C. one culture survived 3 trials out of four, and at 68.3°C. the same culture survived 1 out of 4 heatings.

Sugar, gelatine, serum solids, and fat showed some protective action for bacteria in the order named. Heat-resistant *E. coli* showed a slight pathogenicity when given orally to guinea pigs. Dominick and Lauter liquid media seemed to be more satisfactory than Salle's.

A2. A Differential Stain for Milk Examination. JEAN BROADHURST AND MARY ANN LONG, Teachers College, Columbia University, New York.

A differential stain combining neutral red and brilliant green is suggested for the direct examination of milk. This stain is composed of the following:

Ethyl alcohol, 95%,	54 cc.
Tetrachlorethane	40 cc.
Acetic acid, glacial	6 cc.
Neutral red, powder	1 gm.
Brilliant green, powder	0.5 gm.

This combination acts as a fixative and a fat remover as well as a stain. It, therefore, simplifies greatly the preparation of milk slides for microscopic examination. The milk smear is made as usual, 0.01 cc. of the milk being spread evenly over a prescribed area (1 sq. cm., or preferably 2 sq. cm. in our experience); the slide is then dipped twice quickly in the neutral red brilliant green stain and immediately dipped twice in water and drained. By this brief process the bacteria and white corpuscles are stained red and contrast well with the milky background which appears as a grayish green film. Counts made by this speedy method compare favorably with the counts by the methylene blue method of Breed and Brew. This neutral red brilliant green stain keeps well.

A3. *The Rate of Chemical Change in Milk Brought About by Certain Lactic Acid Streptococci.* C. D. KELLY, New York Agricultural Experiment Station, Geneva.

The proteolytic action of strains of *Streptococcus lactis* (Lister) Löhnis and *Streptococcus cremoris* Orla-Jensen were compared over a period of time in milk containing calcium carbonate and in milk to which no calcium carbonate had been added. The presence of calcium carbonate appeared to increase the hydrolytic action of the organisms on the protein and sugar in the medium. Little difference was noted in the rate of production or in the final amount of acetic acid-, trichloracetic acid-, or phosphotungstic acid-soluble nitrogen produced by these two species.

When the two species of lactic acid organisms were compared with a commercial "starter" it was found that *Streptococcus cremoris* and the "starter" produced approximately the same amount of amino nitrogen (Van Slyke method) when all were incubated under similar conditions. The amount of amino nitrogen found in the *Streptococcus lactis* cultures was less than that produced either by *Streptococcus cremoris* or the "starter."

At the time of inoculation the hydrogen ion concentration of the calcium carbonate milk was pH 6.43. There was a rapid increase in hydrogen ions for two days after which the pH remained more or less constant for 18 days. The lowest reading obtained in the samples taken before shaking was pH 4.78, while in those taken after shaking the readings were close to pH 5.2. At pH 5.2, no significant hydrolysis of the milk proteins takes place when incubation is at 25°C.

A4. The Deterioration of Cream by a Lipolytic Bacterium. J. A. ANDERSON AND J. G. HARDENBERGH, Rutgers University and Walker-Gordon Laboratory Co., Inc.

A peculiar type of cream spoilage is being studied which is characterized by a marked lipolytic action on the butter fat at refrigeration temperatures. After several days, the cream acquires a slightly bitter flavor accompanied by an irritating after effect which resembles the beginning of a sore throat. The causal agent is a gram negative rod resembling certain members of the achromobacter and alcaligenes groups in its reactions on most of the ordinary media. It fails to grow above 34° or 35°C. The only biochemical change worthy of note is the hydrolysis of fats. Closely related bacteria have been isolated from deep well water which, together with the maximum temperature of the organism, indicate that it is of water or soil origin. Quantitative studies of the lipolytic action are now being made. The prevalence of this type of spoilage appears to be of appreciable economic importance.

A5. Bacteriophage as a Precursive Agent in the Development of Organisms Causing Ropy Milk. C. N. STARK AND PAULINE STARK, Cornell University, Ithaca, N. Y.

The most common cause of ropy milk in many localities is the slimy type of *Bact. aerogenes*. Previous results from this laboratory have shown that these ropy types of *Bact. aerogenes* frequently occur in large numbers on dairy feeds and plant materials.

We have found that stock cultures of *Bact. aerogenes* may be easily dissociated into a slimy type which will produce ropiness in sterile milk. This dissociation can be more quickly induced by the addition of an amount of phage too small to cause complete lysis of the culture. The occurrence of aerogenes phage has been demonstrated on lettuce, carrots, corn silks, and other plant tissues.

A6. Comparison Between Bacterial Plate Count of and Acid Development in Pasteurized Milk at 70°F. ARCHIBALD R. WARD AND H. A. HARDING, Dairy Research Bureau, F. O. Adams, Detroit Department of Health Laboratories, and H. G. HARDING, Akron, Ohio.

The object of this study was to determine the relation between the bacterial plate count of pasteurized milk as delivered to the consumer and the rate at which acidity develops in such milk when it is held at 70°F. Since the predominant change in pasteurized milk under such conditions is the development of acidity this is equivalent to studying the relation between the bacterial plate count and the keeping quality of such milk.

The material studied was in quart bottles collected by the Inspectors of the Detroit Department of Health. These bottles were taken from delivery wagons in the early morning, were brought to the Department Laboratory and sampled for fat determination and bacterial plating as usual. The platings were made upon standard agar prepared from Bacto nutrient agar dehydrated. The details of the technic followed the Standard Methods of Milk Analysis of the American Public Health Association.

The remainder of the quart of milk was then distributed among 4 sterile half-pint milk bottles and closed with sterile caps. The bottles were placed in a special Frigidaire cabinet, provided with heating units and a thermoregulator which maintained the temperature at approximately 70°F.

Acidity determinations were made of the milk as it was placed in the temperature chamber. Later, observations were made including titration of acidity, methylene blue determinations and observations from the standpoint of taste and of smell.

These observations failed to show any relation between the bacterial plate counts of the pasteurized milk and the keeping quality when held at 70°F.

A7. *Bacterium acidi-propionici δ and Other Lactate-Fermenting Bacteria of Swiss Cheese.* WILLIAM C. FRAZIER AND HELEN U. WING, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington, D. C.

In the study of eye formation in Swiss cheese, counts made of the numbers of lactate-fermenting bacteria at different stages during the manufacture and ripening have shown that certain tetracocci and small streptobacilli usually predominate through the time of eye formation and the *Bacterium acidi-propionici* type usually predominates after eye formation is complete. Variations in the numbers of propionic or "eye-forming" bacteria added in pure culture may influence the relations of the lactate-fermenters and the consequent set of the cheese.

In experimental cheese an excess of "eye-former" culture usually produced an overset cheese, and excessive growth of eye-formers, especially in the later stages of ripening, tended to cause glass and checking. Coincident with the use of reduced amounts of eye-former culture in a large group of factories, less checking and glass have been experienced than in previous seasons.

When moderate quantities of eye-former culture were used in the cheese, the number of eyes formed, the time of the beginning of eye formation and the time required for the set to become complete were independent of the number of "eye-forming" bacteria added. Characteristic eye formation took place in some cheese when less than 100 *Bact. acidi-propionici* δ bacteria per gram were present at the start of eye formation and little or no increase in numbers had taken place a week later. Such cheese was usually not as sweet in flavor as cheese with more propionic bacteria.

A8. The Influence of Bacterial Growth and Acid Production on the Draining of Swiss Cheese in the Press. LLOYD A. BURKEY AND GEORGE P. SANDERS, Bureau of Dairy Industry, United States Department of Agriculture, Washington, D. C.

It was desirable to learn the differences in rate of the drop in temperature between the outside and inside of the cheese and what influence this might have on the rate of bacterial growth and acid production. Temperatures at the top, edge, and center of the cheese were taken at regular time intervals. At the same time the pH and bacterial count of the curd were determined. A differential bacterial count was made by the direct microscopic method.

A higher growth rate of the starter bacteria was found to occur in the outside portion of freshly made Swiss cheese due to the more rapid cooling of this portion as compared to the inside. The *Lactobacillus casei* cultures, which are active at high temperatures, start growth in the outside of the cheese 2 to 3 hours after it is dipped. However these cultures do not begin growth in the center of the cheese until 3 hours later. This results in an early, high acid development in the outside portion of the cheese. Growth in the inside of the cheese is limited to *Streptococcus thermophilus* for the first 5 hours. A wide ratio of the acid development between the outside and inside of the cheese is believed to cause a checking of the flow of whey due to the knitting together of the cheese particles in the outside portion. This condition, contrary to the principles of good cheese making, results in an inferior product. It is believed this trouble can be remedied by the use of starters which will provide an active growth of *S. thermophilus* in combination with the proper *L. casei* culture. A culture of *S. thermophilus*, referred to as C_3, is believed to be superior to previous cultures used. Similar cultures have been found to make up an essential part of the starters used for making Swiss cheese in New York, Ohio and Wisconsin.

A9. The Control of Mold on the Surface of Cheese. L. A. ROGERS, Bureau of Dairy Industry, Washington, D. C.

The growth of mold on the surface of cheese of the Cheddar type may be entirely prevented by sealing the cheese in a tin can, glass jar, or other suitable container. This has not been successful commercially because the gas produced in the normal ripening of the cheese causes a swelling of the container. This difficulty has been overcome by a simple check valve attached to the can in such a way that the gases formed by the cheese escape without permitting the ingress of air.

A10. The Quality of Commercial Sauerkraut. CARL S. PEDERSON AND C. D. KELLY, New York Agricultural Experiment Station, Geneva.

Samples of sauerkraut have been obtained from packers throughout the United States. Analyses were made of the quality in relation to the chemical and physical characteristics. From the results it is obvious that in many cases the fermentation was abnormal. In many other cases secondary fermentations had occurred. More care in packing and greater use of quantitative methods of testing salt content and acidity by the industry would appear to be necessary to improve quality. The results of this work will appear in a publication from the New York Agricultural Experiment Station.

A11. Souring of Dates by Yeasts. C. R. FELLERS AND J. A. CLAGUE, Massachusetts State College, Amherst.

Spoilage of dates by souring causes considerable losses in the industry. This loss is greatest in wet, rainy seasons when the dates are difficult to mature and dry properly. Sour dates are usually soft-textured and dark in color and possess a peculiar aromatic sour odor and flavor. Though edible, such dates are of very poor quality and are usually discarded. In general, souring occurs when the moisture content exceeds 23 to 25 per cent. Some varieties are more susceptible to souring than others.

Several closely similar types of yeasts were isolated from samples of Iraq, Algerian and California dates which had soured. These yeasts were grown in pure culture and produced typical sour dates when placed in contact with sound, pasteurized dates containing about 25 per cent moisture. Acetobacter was sometimes associated with yeasts on sour dates. Cultural reactions of the organisms were studied.

Souring is effectively controlled by drying dates to a maximum moisture content of approximately 23 per cent and maintaining the fruit during shipment and storage at or preferably below this figure. Pasteurization of dates by holding at 160°F. for 60 minutes at relative humidities about 70 per cent, effectively destroys the date-souring yeasts and serves as an additional control measure.

A12. A Study of the Bacterial Fermentation of Poi. ETHEL K. ALLEN AND O. N. ALLEN, Department of Botany, University of Hawaii, Honolulu, T. H. (Read by title.)

Poi was one of the chief foods of the old Hawaiians and is today a very common food commodity on the markets throughout the Islands because of its nutritional value. It is made by pounding the steamed roots of the taro plant, *Colocasia antiquorum* var. *esculenta* into a smooth paste with the addition of small quantities of water. This starchy product may be eaten immediately, but is most desired after three or four days fermentation. According to Miller steamed taro root has the following chemical composition: water 64 per cent, protein 1.18 per cent, fat by ether extract 0.169 per cent, starch by acid hydrolysis 29.31 per cent, starch by saliva hydrolysis 24.56 per cent, sucrose 1.40 per cent, reducing sugars 0.391 per cent, ash 0.588 per cent, calcium 0.026 per cent and phosphorus 0.06 per cent.

Samples of poi from nine of the principle poi factories in Honolulu were used in these investigations. The acidity of freshly ground taro roots was found to increase from pH 5.8 to pH 3.5 or more after three days incubation at room temperature. In a few cases the acidity of the three-day poi was increased to pH 2.7. These determinations were made by means of the quinhydrone potentiometer. Determinations of the changes in the starch and sugar contents of the poi during fermentation are now in progress.

The fermentation of poi in all present practices is a result of natural fermentation and selectivity of organisms. The flora of the poi samples has been studied by plating on a beef-extract-peptone base plus various carbohydrates. The base plus glucose has proved to be the most satisfactory medium. The flora of the various poi samples with an acidity not greater than pH 4.0 was characterized by very high counts of bacteria and yeasts. Cultural tests have shown this flora to be chiefly that of the colon-aerogenes group. Very few pin-point colonies were detected. Poi older than three days or with an acidity greater than pH 4.0 have shown very few of the colon-aerogenes colonies but instead a predominating abundance of the minute pin-point colonies which together with a much reduced yeast flora seem to be ultimately responsible for the characteristic flavor, texture and acidity of the final product. Organisms characteristic of the various stages of the fermentation have been isolated and studied culturally. Individual pure cultures are being used in various combinations as starters in the making of poi under controlled conditions in the laboratory.

A13. Aberrant Cultures of Rhizobium. LOIS ALMON AND I. L. BALDWIN, University of Wisconsin, Madison.

Work has been done to show that in all probability the organisms which cause nodule formation on leguminous plants may have cultural characteristics markedly different from those which are at present recognized as typical. The most noticeably different character in most of the aberrant cultures is chromogenesis; yellow, orange, or red-brown pigments may be produced.

Evidence to substantiate the identity of these cultures with root nodule bacteria lies in the facts that they may be obtained from filtrates of normal cultures of known purity by the Hauduroy plate technique, or by the cultivation of pure, normal cultures in filtrates of old cultures, and that they have been obtained on several occasions from the nodules of plants grown under controlled conditions, i.e., sterilized medium and bacteria-free seed in cotton plugged bottles, which had been inoculated with pure, normal cultures. They have also appeared spontaneously on certain occasions in laboratory cultures.

So far as is at present known, these cultures do not form nodules. Efforts are being made to change the non-typical forms to typical nodule-producing forms by variations in media, oxygen tension, reaction, temperature, and frequency of transfer. A few normal looking cultures which have been obtained by some of these variations are at present being tested in the greenhouse for nodule production.

A14. The Growth of Rhizobia Upon Rich Nitrogenous Media. A. W. HOFER AND I. L. BALDWIN, University of Wisconsin, Madison.

Marked differences in physiological characters are exhibited between the alfalfa root-nodule organism, *Rhizobium meliloti* and other rhizobia by culture on certain media, rich in nitrogenous constituents. Under suitable conditions the bacteria of the alfalfa group are more vigorous liquefiers of gelatin than are the other rhizobia. Likewise, good growth of the alfalfa bacteria may be secured in certain high nitrogen media which do not permit the growth of other species of rhizobia.

Approximately 75 strains of rhizobia representing 7 cross-inoculation groups were tested for their ability to liquefy a beef-extract, peptone gelatin stab, adjusted to pH 7.0. Inoculation was made from agar slants and the gelatin cultures were incubated at a temperature of 16° to 20°C. for a period of about two months. All of the *Rh. meliloti* cultures gave fair growth and liquefaction of the gelatin. None of the clover cultures, *Rh. trifolii*, liquefied. Although fair growth was secured with certain of the other species, uniform liquefaction of the gelatin did not occur.

The same cultures were inoculated into sheep brain medium (von Hibler) and into veal-infusion peptone broth. All of the *Rh. meliloti* cultures developed in each of these media and no visible evidence of growth was secured with any of the other species.

The veal-infusion peptone may prove valuable as an aid in testing the purity of rhizobia other than the alfalfa organisms, since the rhizobia do not develop in this medium and most other bacteria grow well in it. Tests on a limited number of strains of *B. radiobacter* indicate that this organism gives good growth in this medium.

A15. Variations in the Infective Power Among Strains of Rhizobia.

I. L. Baldwin and A. W. Hofer, University of Wisconsin, Madison.

Marked differences exist in the infective ability of various strains of a single species of rhizobia, and the infectiveness of a strain may be modified by culturing on certain media. Three general lines of evidence are offered in support of these statements.

First, about 20 strains of *Rhizobium japonicum* (isolated from soybeans) have been repeatedly tested, over a period of 4 years, for their ability to cause nodule formation on soybean, (*Soja max*), and cowpea, (*Vigna catjang*). In these tests, both plants were grown in the same pots under carefully controlled conditions. Certain strains produced nodules on both hosts in each test;

other strains always produced nodules on the soybean plants and produced nodules on the cowpea plants in certain tests but failed to do so in other tests; a third group usually produced nodules on soybeans (occasionally failing to do so) and never produced nodules on cowpeas. Less extensive tests with a series of strains of rhizobia isolated from cowpea plants and tested against various species of *Phaseolus* gave similar results.

Second, it is well known that the resistance of the host plant against infection by rhizobia can be modified by varying either the nitrate content of the soil or the photosynthetic conditions under which the plants are grown. Certain strains of *Rhizobium japonicum* produced nodules on soybeans when the plants were grown in a nitrogen free sand under good conditions for photosynthesis, but failed to do so when the photosynthetic conditions were less favorable or when the plants were fertilized with nitrates. Other strains of *Rh. japonicum* caused nodule formation in all of the tests.

Third, the infective ability of certain strains of rhizobia was decreased by long cultivation on certain media and in certain cases the infectiveness apparently was entirely lost. Either nitrogen-free or high nitrogen media at certain times seemed to be effective in bringing about this decrease in infective ability.

A16. The Comparative Growth Rates of Rhizobium meliloti and Rhizobium japonicum. R. H. Walker, D. A. Anderson and P. E. Brown, Iowa State College, Ames.

The growth rates of A and B strains of *R. meliloti* and *R. japonicum* were measured when the organisms were grown in synthetic nitrogen-free media and in similar media containing nitrate nitrogen. Glucose and mannitol were employed as sources of energy. The media were inoculated with 10 cc. of a 24-hour culture of the organisms. The cultures were grown in one-liter round-bottom flasks which were immersed in water held at 28°C. in a Freas constant temperature water bath. The duration of the tests was 20 hours in some cases and 26 hours in others. Dilution and plating of 1 cc. samples were made at intervals of 2 hours. The number of organisms in the samples were determined by the agar plate method. Extreme care was taken to standardize the technic and to eliminate, as nearly as possible, all variable factors.

From the data secured, the velocity coefficient of growth and the generation time were calculated for the period when the organisms were growing logarithmically. Both the A and B strains of *R. meliloti* grew faster in the presence of nitrate nitrogen than in the absence of combined nitrogen in the media supplied with mannitol as a source of energy. In the glucose media the rate of growth of the A strain was slightly higher in the presence of nitrate nitrogen than without it, but the rate of growth of the B strain was slightly higher in the absence of combined nitrogen. Both strains of *R. japonicum* grew faster in the presence of nitrate nitrogen, in both the mannitol and glucose media, than they did without added combined nitrogen.

In all the media, except that with mannitol and no combined nitrogen, the rate of growth of the A strain was higher than that of the B strain of *R. meliloti.* In the tests with the A and B strains of *R. japonicum* just the opposite results were obtained; the B strain grew faster in all media except that with mannitol and no nitrogen.

Neither the differences in the rates of growth of the different strains, nor the differences between the same strains in various media, however, were as large as the differences in rates of growth between the two species. The average generation time for *R. meliloti* in all the tests was 2.74 hours, while for *R. japonicum* it was 7.11 hours. It is apparent that *R. meliloti* reached the end of the logarithmic phase of growth soon after the 14th hour in most of the tests, while *R. japonicum* grew logarithmically throughout the test period of 26 hours. When seeded with approximately the same number of organisms, 400,000 and 520,000 per cubic centimeter for *R. meliloti* and *R. japonicum* respectively, due to the large difference in rate of growth the numbers after 26 hours incubation differed widely, being 74,000,000 and 7,100,000 respectively for the two species. Furthermore, *R. meliloti* had passed the logarithmic growth period while *R. japonicum* was still in this phase of growth.

A17. *The Effects of Nonbeneficial Nodule Bacteria on Austrian Winter Peas.*

LEWIS T. LEONARD AND W. R. DODSON, United States Department of Agriculture, Washington.

Cultures of nodule bacteria were obtained from vigorous and sickly plants of Austrian winter pea collected in a field near Jeanerette, La., and tried several times on this legume in the greenhouse under controlled conditions. The results in general indicate that cultures from sickly plants are practically nonbeneficial while those from the vigorous plants fix nitrogen in accordance with established conception of the relationship between nodule bacteria and legume. Although this would seem to definitely place the responsibility for the poor crops of Austrian winter peas at this location solely on the presence of inefficient nodule organisms in field work, drainage and soil amendments were considered. In spite of the high water table in the territory around Jeanerette, better drainage obtained by ridging the plots had little effect. The soil was practically neutral, and, as would normally be expected, applications of ground oyster shells and basic slag were apparently without effect.

The introduction of previously tested efficient Austrian winter pea nodule bacteria on some of the seed that was planted at Jeanerette last year resulted in a very favorable response, in some instances amounting to around 90 per cent increase over the crop from uninoculated seed. Nodules occurred on uninoculated plants and the fact that this crop was sickly demonstrates the presence of inefficient organisms in this soil.

In this district the only legume of widespread distribution that one would suspect to be related to Austrian winter pea from the standpoint of nodule bacteria, is Louisiana vetch, *Vicia ludoviciana*. Cultures of bacteria from the nodules of this legume produced effects on Austrian winter peas in the greenhouse similar to those by bacteria from sickly plants of winter peas. This result points to a possible cause of the failure of Austrian winter peas at Jeanerette, namely a native nodule organism capable of producing nodules but entirely unsuited for a favorable association with the Austrian winter pea.

A18. The Formation of Pulp and Paper Mill Slimes by Microorganisms. J. R. SANBORN, Research Division, International Paper Company, Glens Falls, N. Y.

In view of the serious economic losses resulting from accumulations of slime in pulp and paper mills, an investigation is being made of some of the causal microörganisms as a possible aid to the discovery of effective methods of control. Though raw water furnishes the most important source of contamination, various means of dissemination and distribution of slime fragments within the mill, foster the general spread of predominating types. Under suitable conditions for development, these microörganisms build up viscous and tenacious growths on the surfaces over which the pulp suspensions flow. The use of a groundwood-glucose-peptone medium facilitated the isolation and study of slime-producing organisms.

Aerobacter aerogenes, Aerobacter cloacae, Pseudomonas viscosa, and species closely resembling *Alcaligenes faecalis, Achromobacter centropunctatum* and *Achromobacter geminum* were prominent among the bacterial isolations. The typical slime-producing filamentous fungi included representatives of the genera Aspergillus (*Aspergillus fumigatus* var., an active destroyer of cellulose), Oöspora, and Actinomyces. The predominating flora of slimes obtained from widely varying sources revealed marked differences.

Chlorination, the generally accepted method of combating slime, has not proven effective in inhibiting the development of slime-forming fungi, though when used in the treatment of the raw water supply, further introduction of contaminating material was usually prevented. The removal of slime accumulations from the system, purification of the water supply, and the exercise of practical mill sanitation are fundamental principles in slime control. In addition, however, each mill presents its own specific slime problems and the remedial measures must be specifically and economically applied.

A19. Thermophilic Fermentation of Beet Pulp. C. H. WERKMAN AND J. STRITAR, Department of Bacteriology, Iowa State College, Ames.

The thermophilic, anaerobic fermentation of beet pulp has been studied in a specially designed apparatus which permitted collection of the gases over mercury. The inoculum consisted of organisms from soil, feces or sludge. Fermentation occurred at 56° to 58°C. at three pH levels, e.g., 5.4, 7.2 and 9.0.

The first process is the fermentation of the sugars, pentosan and pectin, followed by attack on the cellulose. Fermentation of the latter gives rise to the first appearance of methane among the products. Lignin is very resistant to attack. The fermentation at pH 9.0 yielded 0.57 per cent formic acid, pH 7.2 gave only 0.11 per cent whereas pH 5.4 showed no formic acid present. Quantitative analysis of the beet pulp before and after fermentation and of the products were carried out in detail.

A20. The Isolation of Thermophilic Cellulose Fermenting Bacteria.

S. Snieszko, (introduced by E. B. Fred), University of Wisconsin, Madison.

Numerous efforts to obtain active pure cultures of thermophilic cellulose fermenters have been made but the cultures, while satisfactorily active in cellulose fermentation, have never met the critical tests for purity as used by the bacteriologist. Work was therefore continued and a culture has now been obtained which fulfills the requirements for purity and yet retains its power to destroy cellulose. The manner of its isolation is herein described.

Old stock cultures and new enrichment cultures were tested for purity and activity. Three forms are invariably present in microscopic preparations from all cultures even those purified to the highest possible degree, by means of the standard bacteriological methods. Two of these forms grow readily on ordinary culture media and are easily isolated, but do not attack cellulose. The third form is found only in a cellulose medium. It is believed to be the true cellulose fermenter and is normally found in association with the two non-cellulose-fermenting forms. Dilution and microscopic counts have shown that these associated organisms in a cellulose-fermenting culture considerably outnumber the cellulose-fermenting organism. In order to change the ratio between the cellulose fermenter and the contaminants and thus favor the development of the cellulose organism, samples from an actively fermenting culture were taken and dilutions made in sterilized broth. These dilutions (1:10, 1:100, 1:1000) were incubated at 60°C. and every 3 to 4 hours were heated for 15 to 20 minutes in boiling water. After every heating dilutions were made from the broth cultures with sterile water, and these subsequently inoculated into cellulose medium. It was expected that the spores of the cellulose-fermenting organism which do not germinate in this medium would survive the heatings, while the contaminants, which grow well in broth, would be removed by the repeated pasteurization. This was in effect what was observed.

To create suitable conditions for growth in a medium subjected to long sterilization, one series of tubes was treated with a suspension of a pure yeast culture. During a period of two months since isolation, growth of the cellulose fermenting organism has been greatly stimulated by this addition of yeast.

The cultures obtained in this manner appear uniform in microscopic preparations, are free from contaminants detectable by standard bacteriological methods and grow only on media containing cellulose. The pure culture is not as active as the crude culture in fermenting cellulose.

A21. The Hemicellulose-decomposing Bacteria. A. Geoffrey Norman (introduced by W. H. Peterson), University of Wisconsin, Madison.

The decomposition of hemicelluloses by bacteria has until recently received little attention, partly because of the structural heterogeneity of these substances, and partly because their preparation in a pure condition and their subsequent estimation present no little difficulty. Though there are some cases in which the sugar units concerned are homogeneous, in general both a hexose and a pentose, and frequently also, a hexose aldehyde monocarboxylic acid, or uronic acid, are involved. The linkage from a chemical point of view is strong, and fairly resistant to mild hydrolysis. Since the sugar units are of unequal availability to most organisms, there is the possibility of the removal of a portion of the molecule, and the accumulation of a more resistant residue.

Enrichment cultures made from soil and manure will ferment fairly readily and rapidly the structurally heterogeneous type of hemicellulose, as typified in this work by the A and B fractions from oat straw. Fermentation takes place even at 65°, though at 25° to 35°C. it is more rapid. From such enrichment cultures a number of strains which would develop on hemicellulose-agar media, was isolated. The mesophilic group consisted mainly of aerobic spore-forming rods, and facultative non-spore formers of the Achromobacter group. The thermophilic organisms were not identified. They were long slender rods, with and without spores, showing marked pleomorphism, and consequently of somewhat doubtful purity.

The fermentation characteristics of these cultures were tested. They developed readily on an extremely wide range of carbohydrates but produced little acid. A number of synthetic disaccharide acids was employed in an attempt to obtain some information as to the type of linkage hydrolysed. Lactobionic acid was utilised by practically all strains, and maltobionic acid by many. Gum arabic, mesquite gum, and tetra-galacturonic acid were fermented, though rather weakly.

However, fermentation of complex hemicelluloses in liquid medium was found to proceed only slowly, and none of the cultures could validly be described as very active. It may be that the decomposition of such polysaccharides is most satisfactorily accomplished by organisms in association, as in the fermentation of cellulose, or, more probably, that the conditions of growth in a liquid medium are less suitable for this type of organism than those ordinarily existing in soil.

A22. The Effect of Associated Growth on the Forms of Lactic Acid Produced by Certain Bacteria. E. L. TATUM, E. B. FRED AND W. H. PETERSON, University of Wisconsin, Madison.

The optical activity of the lactic acid produced by certain bacteria is dependent not only on the nature of the organism but also on the presence of associated microörganisms.

In this investigation there were used two strains of *L. leichmanni*, one of *Leuconostoc pleofructi* and one of *L. delbrücki No. 4* all of which produce exclusively levo-lactic acid. In addition three cultures of dextro-lactic-acid-forming bacteria were included, *Str. lactis*, *L. casei*, and *L. delbrücki No. 3*. When grown in association with the non-lactic-acid-forming organism *Cl. acetobutylicum*, (the industrial acetone butyl alcohol organism) all of these true lactic organisms were found to form inactive lactic acid. Other non-lactic-acid-producing organisms, i.e., yeasts and butyric acid bacteria, did not bring about this effect when grown in association with the lactic organisms above.

It was shown that this effect is caused by some heat labile substance produced by *Cl. acetobutylicum* which does not diffuse through a viscose membrane. An intimate contact between the two types of cells seems to be necessary to obtain the production of inactive lactic acid. Collodion membranes were penetrated by the organisms.

The mechanism has been suggested as involving a disarrangement of the enzymatic system of the lactic organism through the activities of *Cl. acetobutylicum.*

A23. Some Factors Involved in the Biological Production of Acetone and Butyl Alcohol. LOUIS WEINSTEIN AND LEO F. RETTGER, Department of Bacteriology, Yale University, New Haven, Conn.

Six strains of *Clostridium acetobutylicum* (Weizmann) were used in this work. A bacteriological study of the organisms showed that they possessed essentially the same morphology and cultural and physiological properties originally described by Weyer and Rettger (1927). A diminution in solvents-producing power of the organisms, due to storage for 5 years in sealed tubes, was overcome by a series of alternate "pasteurizations" and cultivations in corn mash.

Studies of the acidity curves from various carbohydrates in Robinson's semi-synthetic medium and the solvents production from them revealed that the curves were abnormal, a fact which could be correlated with the failure of the organisms to produce butyl alcohol from these sugars.

An inquiry was made into the effect of basic medium, peptone concentration, colloidal material, hydrogen ion concentration, oxygen tension, oils and proteins, and it was concluded that an alcohol-soluble protein is necessary for the production of appreciable amounts of butyl alcohol in Robinson's semi-synthetic medium.

Various natural materials such as cottonseed hulls, hard and soft wood dusts, peanut hulls and corn cobs were hydrolyzed with acids, the hydrolysates neutralized and allowed to undergo fermentation with the solvents-producing organisms. Normal amounts of acetone were formed, but practically no butyl alcohol. The addition of a prolamine-containing material in the form of yellow corn stimulated the production of normal amounts of both solvents, the total yield of these neutral products being larger than that obtained from the corn or the natural material alone.

Experimental proof was obtained that the prolamine does not act as a catalyst in the reaction involved in the formation of butanol, and that the alcohol is not derived from the protein itself. One explanation for the definite influence of alcohol-soluble proteins on the production of butyl alcohol from carbohydrates in Robinson's semi-synthetic medium will probably rest on a physico-chemical basis.

A24. The Microflora of Old Volcanic Ash with Special Reference to Nitrogen-fixing Bacteria. NATHAN R. SMITH, United States Department of Agriculture, Washington, D. C.

Samples of volcanic ash from the region of Mt. Katmai, Alaska, were collected in 1930, 18 years after the eruption, by Prof. R. F. Griggs, and brought to Washington, D. C., where the microflora was studied by the author. In the bare ash, no fungi or actinomycetes were found. The bacteria numbered about 150,000 per gram, consisting mainly of gram-negative small rods. None of the usual nitrogen-fixing bacteria were found.

In the ash on which a carpet of liverworts (Jungermaniaceae) were growing, fungi were found in small numbers but no actinomycetes. The bacteria in the surface layer varied from 65,000 to 300,000; one inch below the liverwort, about 900,000; and 6 inches below, 1,400,000. *B. radiobacter* was easily isolated from the liverwort layer and to a less degree from underneath the layer. Nitrogen-fixing experiments using mixed and pure cultures of *B. radiobacter* failed to show any appreciable gain in nitrogen. *Azotobacter* was never found. It is therefore concluded that if nitrogen-fixation occurs in volcanic ash, it probably is not due to the microflora.

A25. *On the Occurrence of Microörganisms in Peat Bogs and Their Rôle in Peat Formation.* SELMAN A. WAKSMAN AND E. R. PURVIS, Agricultural Experiment Station, New Brunswick, New Jersey.

The occurrence of bacteria in peat has attracted considerable attention, due to the fact that it has been commonly assumed that the processes of peat formation are largely chemical and that microörganisms do not play any part in these processes, after the peat has been laid down. A detailed study of the occurrence of aerobic and anaerobic bacteria in the different layers of low moor and highmoor peat profiles has revealed the fact that although certain layers of peat, such as sphagnum peat, may be very poor in bacteria, due to the fact that the chemical complexes of the sphagnum are highly resistant to decomposition, other layers made up of forest and sedge peat contain a great abundance of bacteria, independent of the age of the particular layer. The study of the undisturbed highmoor peats in Maine has shown that while the surface layers may contain 200,000 to 2,000,000 bacteria per gram of peat on a dry basis, at a depth of 5 meters there will be found 50,000,000 or more bacteria per gram of dry peat. The presence of cellulose-decomposing bacteria, as well as the association of considerable gas evolution in those layers where the bacteria are most abundant tend further to emphasize the fact that these bacteria are active in the processes of transformation of the peat, even centuries after the peat has been laid down. Most of these bacteria are facultative anaerobes and are capable of growing at fairly high acidity.

A26. *Nitrogen Metabolism of Bacteria as Affected by Colloids and Replaceable Bases in Soil.* H. J. CONN, AGRICULTURAL Experiment Station, Geneva, N. Y.

It is now a matter of common belief that the low productivity of acid soils is due not only to the direct influence of the reaction but perhaps to a greater extent to the high degree of adsorption of soil bases in such soils. Certain recent papers try to show that the same factor controls bacterial nutrition in the soil; but they do not establish the fact at all conclusively. The present paper offers a little more evidence on the subject. It is shown that certain non-spore-forming soil bacteria (humifiers) fail to grow in certain soils high in total nitrogen unless, in addition to lime, some nitrogen compound or some salt (or hydroxide) of a strong base be added to the soil. This suggests that these soils are deficient in nitrogen available to the bacteria, but that the nitrogen present is made available by the non-nitrogenous salts added. It is noticeable that the soils in which this effect is pronounced are ones with a high ratio of colloids to calcium. That the nitrogen present exists in some form (e.g., ammonium salts) adsorbed by the soil colloids and capable of being replaced by the salts of strong bases is not at all improbable; and the data at hand seem to give further evidence that bacterial nutrition in soil is influenced by the exchangeable bases present.

A27. *The Soil Plaque Azotobacter Test for Soil Deficiency.* DAN. H. JONES, Ontario Agricultural College, Guelph, Canada.

In an attempt to estimate the value of the azotobacter soil plaque method of testing for soil deficiency, 21 samples of soil were tested by this method. The results were compared with crop returns.

The soils tested fall into 3 groups. The first group includes the first 12 samples of the series. These samples were taken from check plots to which no phosphate fertilizers had been applied. The plots were located in fields that had received applications of phosphate fertilizers in recent years, previous to being sown with various crops. These fields are located in Norfolk, Simcoe, Halton, Peel, Huron and Perth Counties, of Ontario, and represent a diversity of soil types.

The results of the test indicated that every sample needed phosphate for azotobacter colony development. The crop returns from the check plots from which the samples were taken when compared with the crop returns from the fields treated with phosphate fertilizers showed that marked benefits had accrued from the phosphate treatment. It would, therefore, appear that the results of the azotobacter soil plaque test in this case showed a correlation with crop returns.

The second group includes soil samples 13 to 19 inclusive. These were all acid clay soils from Welland County. They had been variously treated with chemical fertilizers, including superphosphate, tricalcic phosphate, lime, sulphur and complete fertilizer, twice during the preceding five years.

The azotobacter soil plaque test indicated that notwithstanding that fertilizer had been applied previous to cropping every sample required phosphate to enable azotobacter colonies to develop, just as in the case of the samples of the first group. Chemical determinations showed that the available P content of these soils was no greater on the average than that of the soils in Group I. It would, therefore, appear that the phosphate added to these plots as fertilizer had been all utilized by the growing crops except in the case where tricalcic phosphate was used, which, not being so readily soluble as the superphosphate, the crops had not so much benefited by it as in the case of the superphosphate; and more of it was left as a residue in the soil. All things considered, there would appear to be a reasonably satisfactory correlation of the results of the azotobacter soil plaque test and crop returns in this group of soils, as was found in the first group.

Group III consists of only 2 samples of muck soils from a reclaimed swamp area in York County. One of these has been under cultivation and crop for 4 years being heavily fertilized with phosphate fertilizer. The azotobacter soil plaque test from this sample showed a heavy azotobacter colony development on the check plaque as well as on the plaque to which phosphate was applied, thereby indicating that this soil did not need phosphates. As the last crop of lettuce from this soil had been very poor it was evident that some factor other than lack of phosphates was the cause of the trouble.

The other sample was from an adjoining area that had only just been reclaimed and was under cultivation for the first time. No fertilizers had been applied. No azotobacter colonies developed in the check plaque, but 3 azotobacter colonies developed on the plaque to which phosphate was added, thus showing that even in the newly reclaimed swamp soil a very few azotobacter occurred, but that owing to lack of phosphate they could not flourish.

As 5 cc. of a 3 per cent solution of K_2HPO_4 added to 50 grams of soil, as in the test, is the equivalent of 15,281 pounds of superphosphate per acre, which is 38 times the amount usually recommended for a grain crop, 4 plaques were prepared to which 5 cc., 2.5 cc., 1 cc., and 0.1 cc. of the phosphate solution was added, to determine if smaller quantities than that called for in the test would be satisfactory. The azotobacter colony growth on these plaques was excellent, fair, poor and very poor, respectively; thus showing that the large quantities of phosphate are essential for good azotobacter colony growth.

A28. Notes on the Association of Microörganisms and Roots.

CHARLES THOM AND HARRY HUMFELD, United States Department of Agriculture, Washington, D. C.

Recent studies of the rhizosphere (Starkey et al.) indicate larger numbers of microörganisms in the ball of earth enclosing the root system than in areas not penetrated by roots.

In the work reported here the first sample consisted of soil outside the root zone. The root and adhering soil were dug up and all loose earth falling away readily was discarded. The second sample was then taken from soil dislodged by vigorous shaking. The third sample consisted of the fibrous portion of root system with particles of earth adhering closely. The kind of results obtained are typified by such figures as follow. In a sample involving vetch, the total plate count outside the root zone was 36 million per gram; soil in contact with roots, 56 million per gram; the fibrous roots, 530 million per gram. In a sample from a rye field, the soil had 70 million; soil in contact with roots, 125 million; the roots themselves, 750 million. Counts of fungous colonies on plates from corn on different soils gave Caribou loam, (pH 4.5), 145,000 per gram; roots, 4,100,000; Keyport clay loam, (pH 5.9), soil 105,000; roots 555,000; Carrington loam, (pH 7), 141,000, roots 700,000; Houston clay, (pH 7.8), 155,000, roots 300,000.

Microscopic examination of fibrous roots of apparently healthy plants shows epidermal cells and cortical parenchyma abundantly infested with mold hyphae, some as wefts and masses, again as widely ramifying filaments. Bacteria were abundant, filling some cells, absent or few in others.

A29. Note Upon the Distribution of Dictyostelium and Other Slime Molds in Soil. CHARLES THOM AND KENNETH RAPER, United States Department of Agriculture, Washington, D. C.

Slime molds such as Dictyostelium and Polysphondylium have been regarded as comparatively rare curiosities in the horizon zone between animals and plants. Even after Krzemieniewski in Poland, and Harper in New York, began to find myxomycetes in soil, it was commonly assumed that they were occasional and probably developed only on dung or on special organic substrates.

Then, the plasmodia of the myxomycetes began to show up in cultures from soil in the Bureau of Chemistry and Soils. Upon favorable culture substrates, amoeboid organisms have appeared in considerable variety and in large numbers, in samples from localities in the District of Columbia, Maryland, Virginia, North Carolina, West Virginia, Tennessee, Kentucky, Ohio, Indiana, Illinois, North Dakota, Colorado and Utah. Plasmodia have developed in samples from most of these states although not all of the samples tested developed either the plasmodia or the fruit bodies of slime molds. Dictyostelium has already appeared in the cultures from Colorado, Indiana, Maryland, Nebraska, New York, North Dakota, Tennessee, Utah and Virginia. It develops readily in culture and has been found from earth samples at various depths under tobacco and blue grass, from decaying plant materials including green stems, weeds and rotting leaves in the forest, and from stems of ragweeds, Erigeron and grasses standing in the field during the cold moist periods of the year. Organisms of this group must then be designated as common and should be included in every study of a soil microbiological complex.

A30. *Mushroom Nutrition: A Group of Problems in Microbiology.*

Selman A. Waksman and W. Nissen, Agricultural Experiment Station, New Brunswick, New Jersey.

Horse manure, after it has been composted for a considerable period of time, has commonly been used as a substrate for the growth of the cultivated mushroom. However, very little is known as to the reasons for composting the manure and as to the specific mineral complexes in the manure upon which the mushroom feeds. A knowledge of these processes was not essential for the practical grower who has followed the procedures laid down by former generations and developed in a practical way. However, when it became necessary to substitute an artificial compost for that of horse manure, it became essential to determine what processes should be favored and what complexes are needed in order to produce a substrate favorable for the growth of the mushroom.

As a result of studies carried out both in pure cultures and in straw composts, the following facts were established: (1) That in the decompostion of horse manure there is a great reduction of water-soluble substances. (2) That there is an accumulation of proteins, largely of microbial origin. (3) That there is a considerable reduction of cellulose and hemicelluloses, which contribute largely to the heat evolution in the process of composting of the manure. (4) That there is an accumulation of lignin and lignin-like complexes. (5) That there is a great increase in the ash content of the composts, due to the considerable loss of the organic complexes in the process of decomposition. These processes seem to be highly essential for the production of a medium favorable for the growth of the mushroom, since the latter seems to feed primarily upon the proteins and lignins of the composts and to a less extent upon the residual cellulose and hemicelluloses.

With this knowledge in mind, an artificial compost can be prepared which should offer the same conditions for the growth of the mushroom as the compost of horse manure.

MEDICAL BACTERIOLOGY, IMMUNOLOGY AND COMPARATIVE PATHOLOGY

Hemolytic Streptococci

Introductory Remarks. J. HOWARD BROWN.

M1. The Antigenic Activity of Hemolytic Streptococci From Different Types of Infection. MARY W. WHEELER, Division of Laboratories and Research, State Department of Health, Albany, New York.

Studies of the antigenic activity of a number of strains of hemolytic streptococci from cases of scarlet fever, erysipelas and epidemic septic sore throat, for the purpose of selecting the strain or strains most suitable for use in the production of a highly potent therapeutic serum effective against the different types of streptococcus infections, afforded a striking demonstration of the complex nature of the streptococcus toxins and antitoxins. Sera produced by certain strains were broadly valent and equally potent against the toxins of the majority of strains from all sources. On the other hand, marked differences in the nature of toxins neutralized by a serum of such broad valency were apparent when their antigenic activities were investigated. Sera produced against certain of these were equally potent against the homologous toxin and toxins of a number of heterologous strains. Other sera were highly potent against the homologous toxin but their activity against different heterologous toxins varied. One serum was active against the homologous toxin only. A mixture of two monovalent sera produced against toxins of different antigenic activity or a polyvalent serum produced by immunization of animals with these same toxins was active against toxins not neutralized by either monovalent serum alone. This ability of one serum or toxin to supplement the action of another did not appear to be related in any way to the origin of the strain.

M2. Agglutination of Hemolytic Streptococci. J. HOWARD MUELLER AND KATHARINE S. KLISE (by invitation), Harvard Medical School, Boston.

An attempt has been made to work out a method for agglutinative classification of hemolytic streptococci which will be simple enough to be of service in throat carrier determinations during outbreaks of surgical or puerperal sepsis, scarlet fever, etc. In our experience, the percentage of hospital nurses and staff carrying hemolytic streptococci is always high, (27 to 40 per cent) representing many heterologous agglutinative groups. If a focus of infection appears, it is quite impossible to say which of these carriers may be involved with the particular virulent strain. The very heterogeneity of this group of organisms should be of aid here, provided a sufficiently rapid and reliable method is available and a fairly large supply of agglutinative sera is on hand. Complete proof of identity, depending on absorption experiments, would not be essential for the practical measures taken to check an outbreak.

To a point, the work has been successful. By the method described, hemolytic streptococci can be agglutinated very satisfactorily, and spontaneous agglutination practically eliminated. The stumbling block to completion of the study and its practical application is our inability to obtain satisfactory agglutinating sera for about one-third of the strains we have worked with. Until this difficulty can be overcome, we are unable to put the procedure to thorough practical test, but we have already had very definite evidence in one hospital outbreak of scarlet fever that it has possibilities. Provided a serum agglutinating the strain from the case or cases is available, it is possible to complete the agglutinations on all carriers within three days of undertaking the work. If the original throat plates are good enough to obtain pure fishings directly, the time can be reduced to two days.

(This work was aided by a grant from the DeLamar Mobile Research Fund.)

M3. Agglutination as a Means of Differentiating Certain Types of Streptococcus and Leuconostoc. G. J. HUCKER, New York Agricultural Experiment Station, Geneva.

A study has been made from the standpoint of agglutination and agglutination absorption of the relationships of certain strains of the genera *Streptococcus* and *Leuconostoc* with particular emphasis upon the relationships of the saprophytic types. The thirty-six strains used for the study were secured from a variety of sources and were selected with the thought of studying a representative series of authentic strains of these two genera rather than a study of a large number of freshly isolated types. No effort was made to form serological groupings.

It was found that agglutination and agglutinin absorption were not useful in all cases in differentiating certain species of the streptococci due in a number of instances to a decided strain specificity.

Of the saprophytic types *Streptococcus lactis* (Lister) Löhnis, *S. cremoris* Orla-Jensen and *S. thermophilus* Orla-Jensen appeared to be distinct from an agglutination standpoint. *S. glycerinaceus* Orla-Jensen was found to be closely related to and possibly identical with the gelatin liquefying streptococci. *Streptococcus faecium* Orla-Jensen serum was found to agglutinate strains of *Streptococcus lactis.*

The saprophytic streptococci did not give cross reactions with the more parasitic members of the genus. *Leuconostoc dextranicus* (Beijerinck) Hucker and Pederson and *Leuconostoc mesenteroides* (Cienkowski) Van Tieghem showed cross reactions to a high degree with sera prepared from many strains of parasitic as well as saprophytic streptococci.

M4. The Use of Chinchilla Rabbits for the Titration of Streptococcal Toxin. F. H. Fraser and M. A. Ross (by invitation), Connaught Laboratories, University of Toronto, Canada.

Chinchilla rabbits have been found to be susceptible to both intracutaneous and intravenous doses of streptococcal toxin; the latter produces characteristic symptoms and death with well defined pathological changes.

The degree of accuracy attainable in a lethal test of a toxin depends on the variation in the individual susceptibility of the test animal and can be determined from the slope of a curve obtained by plotting the deaths per cent against the logarithm of the dose of toxin. Such a curve obtained for an acetone precipitate of streptococcal toxin indicated that two toxins, of which one was two-thirds the strength of the other, could be differentiated in 95 per cent of the trials when each toxin was injected into a group of 30 animals.

Within the limits of the test, no deterioration of the acetone precipitate occurred during three months storage.

An attempt to obtain a similar curve for mixtures of toxin and antitoxin is described.

M5. Some Toxins of Scarlatinal Streptococci. SANFORD B. HOOKER AND EDNA M. FOLLENSBY, Department of Immunology, Evans Memorial, Boston, Mass.

Several qualitatively different active substances, capable in high dilution of evoking skin reactions, are demonstrable in a collection of filtrates from scarlatinal streptococci. Of these, two, which we regard as toxins, have been sharply differentiated by comparing their physical, chemical, skin-reactive, and antigenic properties, and by specific neutralization. The one, "A," is the Dick toxin whose relation to scarlet fever has been firmly established. The other, "B," is being investigated with the object of learning its pathogenic significance.

"B" is clearly distinguishable from the "nucleoprotein" studied by Ando. Among many others, the Dochez strains, N. Y. 5, etc., produce both "A" and "B;" some strains produce one, or the other, or neither. Among 100 strains from convalescents, 10 failed to produce "A" and 10 did not produce "B;" 45 produced "A" in concentration of 1000 or more s.t.d. per milliliter and 68 produced "B" in similar, usually higher, potency. The same proportions have been obtained in tests upon the strains from 55 patients in the early stage of the disease. The ability, then, to form toxin "B" is commonly possessed by scarlatinal streptococci.

About 40 per cent of several hundred adults reacted to 1 s.t.d. of "A," and 5 per cent to "B." Half of the latter reacted to both "A" and "B". Persons artificially immunized by "A" still react to "B." Of 55 patients tested early in the disease, 22 were positive to 5 s.t.d. of "B" toxin; under the same conditions, "A" reactors constituted likewise about 40 per cent. Search for "B" toxin in the blood and urine of appropriately selected patients has been thus far unsuccessful; "B" toxin has not yet been definitely implicated in the pathogenesis of scarlet fever. It has explanatory possibilities in connection with numerous discrepancies which appear in studies of the mechanism of the disease. There is some circumstantial evidence that additional toxins exist.

M6. Comparison of Different Strains of Typical and Atypical Pneumococci and Streptococci.

GRACE M. SICKLES, Division of Laboratories and Research, State Department of Health, Albany, New York.

A study was made of the differences in cellular susceptibility, as shown by the maximal limits of growth and viability, of pneumococcus strains which had become atypical in the tissues of horses undergoing immunization, in comparison with the typical strains from which they were derived, a typical strain obtained from one of them by mouse passage, typical and atypical pneumococci from other sources and non-hemolytic streptococci. All of the strains classified as pneumococci were bile soluble. None of the strains classified as streptococci was soluble in bile.

The final hydrogen ion concentration, fermentation of carbohydrates, peroxide production and methemoglobin formation were also noted.

The maximal limits of growth and other characters of the typical, atypical and reverted pneumococci of the same strain were very similar. None of the pneumococcus strains grew at temperatures above 42°C. and only one strain grew at that temperature. This strain was alive after 20 to 24 hours at 43°C. and sometimes at 43.5°C. None of the other pneumococcus strains used was alive after 20 to 24 hours at 42°C.

The maximum limits of growth of the non-hemolytic streptococci varied between 42° and 49°C. Marked variations were also observed in the other characters studied.

M7. Epidemics of Septic Sore Throat of Milk Origin: Present Status of Bacteriology and Epidemiology. D. J. DAVIS, University of Illinois, College of Medicine, Chicago.

From our studies of septic sore throat, the following points seem to be established:

1. The epidemics are sudden, sharp and severe, indicating a massive dosage of the infecting agent.

2. The sick are largely limited to users of one milk supply. There are relatively few contact infections.

3. Epidemics rapidly recede when the infected milk supply is cut off or the milk pasteurized.

4. Usually one cow is found with an udder infected with an encapsulated hemolytic streptococcus. This organism produces large mucoid, watery, spreading colonies growing especially well on ascites blood agar. These organisms were observed in the Chicago epidemic of 1910–1911 and have become known since then as the *Str. epidemicus.*

5. Experiments indicate that when these streptococci are implanted in the cow's teat, they will rapidly ascend the duct and localize in the udder, causing mastitis. They may continue there for long periods thus giving rise to the carrier state. The udder may or may not reveal gross physical changes.

6. Similar encapsulated streptococci are uncommon in the throats of normal persons (less than 1 per cent) but more common in infected throats (12 per cent of tonsils). Sporadic cases of septic sore throat indistinguishable from epidemic cases are occasionally seen. From such throats encapsulated streptococci of the epidemicus type may be found.

7. These encapsulated streptococci produce toxins which give rise in animals to specific neutralizing antiserum. The toxins cause specific skin reactions in susceptible persons (differing from scarlet fever toxin). A study of several strains indicates that they are heterogeneous by the agglutination test. Agglutination tests however are often unreliable for encapsulated organisms. We have not as yet compared the toxin antitoxin properties of a number of different strains.

8. From a study of many epidemics of septic sore throat, it

appears that not infrequently cases of scarlet fever and erysipelas occur as complications. There is marked variation in this respect. At times scarlet fever and skin rashes are relatively common. Again they may be rare or absent and erysipelas relatively common. On the other hand it is well known that sore throat without rash is common during epidemics of scarlet fever.

9. Strains of streptococci classed as scarlet fever or erysipelas organisms on the basis of skin and other tests at times develop capsules and grow quite like strains of *Str. epidemicus*. Such strains may be the cause of milk-borne scarlet fever. The variability in these clinical manifestations together with the variability in properties of streptococci from epidemics of septic sore throat, scarlet fever and erysipelas suggest the possibility of a variable toxigenic and serologic property.

10. A study of the world distribution of epidemics of septic sore throat of milk origin reveals three general localities: namely, (*a*) Great Britain, (*b*) Scandinavia and Denmark and (*c*) United States and Canada. In other parts of the world the methods of treating milk supplies may explain their freedom from infections.

M8. Carriers of Streptococcus epidemicus. W. D. FROST, Department of Agricultural Bacteriology, University of Wisconsin, Madison.

The work on which this paper is based was undertaken for the specific purpose of detecting carriers in both man and cow on certain large milk producing farms in Wisconsin and Illinois.

A period of monthly examinations covering four years for throat cultures and six years for milk samples is reported on.

The throat swabs were made by the attending physicians and sent to the laboratory where they were usually received in 4 hours, however for two of the farms it was a 24-hour trip. When received, they were first rubbed on Loeffler's blood serum and then 2 cc. of veal broth was poured on them and they were allowed to stand 2 hours at 37°C., when a loopful was used to make poured blood agar plates. After an incubation period of 24 and 48 hours, representatives of all the *beta* hemolytic colonies were picked into broth, tested for hemolytic titer and if pronounced, put onto freshly made blood agar slopes and 8 to 12 hours later examined for capsules. Positive strains were then run against the following test substances; dextrose, lactose, saccharose, mannitol, salicin and sodium hippurate. A strain was regarded as *Streptococcus epidemicus* if it possessed capsules, produced large and moist surface colonies on blood agar plates and the "giant colonies" both on the surface and the bottom, i.e., between the agar and the glass, when additional serum or ascites fluid was used as suggested by Pilot and Davis, hemolyzed in the test tube, fermented all of the test substances except mannitol and sodium hippurate and was not more acid in dextrose than is represented by pH 4.9. Approximately 4750 throat cultures have been examined from about 750 different men; 19 carriers have been found, 16 of them on the special farms; 2, the probable causes of 2 of Wisconsin's 3 epidemics of septic sore throat; one of our laboratory workers, and a school child. Only 3 of these carriers gave any history of illness. One, the milker of the incriminated cow in the Baraboo epidemic, probably had typical septic sore throat; another, a dairy worker, and the laboratory worker had a mild tonsillitis. The rest were without sign or symptom of disease.

Composite samples from 10 cows each were plated on blood agar plates in dilutions of 1:20 and suspicious colonies picked and examined as noted for the throat cultures. Approximately 10,000 samples have been analyzed from about 3000 different cows; 8 carrier cows have been found. One of these had an evident mastitis when found and 3 others developed a mastitis later. The other 4 cows had an extremely mild, chronic mastitis which would have been detected only by the most careful analysis through a physical examination of the milk or a bacteriological analysis.

Our knowledge of the distribution and nature of this new disease, "*Epidemicus Mastitis*" has been greatly extended through samples of gargety milk sent to the Veterinary Department of the University and the new Control Laboratory of the State Department of Agriculture and Markets. In this way 58 additional cows have been found in 17 different herds and in 14 different counties of Wisconsin. Four of the badly infected herds had 5, 6, 7 and 13 such cows respectively at one time, which constituted from 20 to 48 per cent of these herds.

M9. Streptococci Isolated from Excised Tonsils and Post-tonsillectomy Blood Culture. II. JOSEPHINE S. PRATT AND MARY S. B. GAILLARD, Fifth Avenue Hospital, New York.

Alpha streptococci appeared in the majority of 325 tonsils, the predominating types being *S. salivarius*, *S. ignavus* and *S. mitis*. The predominating beta types were *S. pyogenes*, *S. subacidus* and *S. anginosus*.

In 600 blood cultures taken from 300 children before and after tonsillectomy, growth was present in 51 of those taken before operation and in 42 of those taken after operation. *Streptococcus viridans* was found in one case, a post-operation culture (0.3 per cent).

The heterogeneity of the streptococci found in the interior of excised tonsils is noted, and the question is raised as to the true specificity of the groups among the streptococci found.

Meningococcus and Meningitis

Some General Considerations Indicating Lines of Investigation of the Mechanism of Meningococcus Infection. E. G. D. MURRAY, Department of Bacteriology, McGill University, Montreal, Que.

We cannot hope to obtain a complete understanding of the physiology and behavior of the meningococcus if we confine our investigations to organisms completely removed from their natural environment. A proof of the insufficiency of our knowledge is our inability to forestall generalized infection (septicemia and meningitis, etc.) and our inability to treat such cases effectively. We are still ignorant of the mechanisms involved in invasion and localization and of the reasons why temporary and chronic carriers have a measure of protection. It is insufficient to cloak our ignorance by the use of terms such as "strains of increased virulence" and "susceptible individuals" and "natural resistance."

The actual disease is the lesion, which is the reaction of the host to the infection, not the presence of the meningococcus in the tissues, and some advantages may be gained by not confusing these two processes; but, as they may not be mutually antagonistic, both must be studied and correlated. It is not unreasonable to hold that our immediate concern is to understand the process of infection, for if this can be prevented or minimized the lesion will be correspondingly avoided or reduced.

One of the sets of conditions I think worthy of further study is that covered by the term "Virulence" which, to my way of thinking, involves the efficiency of the defensive mechanisms of the host just as much as the aggressive powers of the parasite; and the degree of virulence we measure may therefore be regarded as the resultant of these two sets of forces. On the one hand we have the mechanism whereby the parasitic potentiality of the organism, contributing to virulence, is increased or diminished experimentally, excluding changes which take place with sub-culture. To some extent this appears to be related to a selective interaction between the leucocytes or their products and the cocci, and we can find a partial expression of it by the degree of resistance to phagocytosis, as well as by the power to kill experimental animals, exhibited by the cultures. There is a possibility of this process occurring in the carrier and in the generalized infection (case); and the demonstration of its operation under these conditions would be very illuminating and take us out of the realms of speculation. Its operation in the cerebrospinal fluid of cases of meningitis might well account for certain clinical manifestations of the disease and it is suggestive that cultures isolated from a single sample of cerebrospinal fluid at different times may vary in their exhibition of virulence. It is possible that cultures possessing a marked parasitic potentiality have a decided influence on active immunization and this may be worthy of consideration in the production of therapeutic serum. On the other hand, we have conditions contributing to the susceptibility or resistance of the host such as those determining the nature of the local reaction, the great rapidity with which invasion appears to take place, the factors controlling phagocytosis and lysis of the cocci and the variation exhibited by the clotted blood of different persons to support the growth of the meningococcus. It is possible to cause variation in the apparent virulence of a strain by interfering with phagocytosis in experimental animals and it is probable that an understanding of this and of the factors involved in the other host reactions may lead to important conceptions. In all these considerations we must bear in mind that the meningococcus is an organism of relatively low pathogenicity compared to other bacteria, in spite of the danger we might expect from its frequent localization in the meninges; and it would be interesting to be able to account for this peculiarity.

Considerable uncertainty still prevails in relation to the production of toxin by the meningococcus, although toxic substances can be extracted from the cells. This exhibits a characteristic degeneration and there is some evidence that it can subsequently be restored to full potency under certain conditions which have not been accurately defined so far. This toxin does not appear to immunize readily and the available evidence of its neutralization by immune serum is not of the most convincing character. Certainly it is lethal to mice and it is probable that in many instances of attempted infection with meningococcus cultures the death of the animal can reasonably be ascribed to this toxin; nevertheless, we have no indication of its mode of action. There is some evidence that the exhibition of virulence by cultures of the meningococcus is independent of their power to produce this toxin.

In view of the findings of many workers who have investigated other organisms, there is a need for information on possible variants of the meningococcus of a rough and smooth nature and it is possible that variants of these kinds might account for certain observations which have been made in relation to both the toxin production and the infective power of a culture.

It has long been known that the agglutinating antigenic value of cultures of meningococcus changes with the time that the strains have been maintained and there is no reason to suppose that similar variations might not occur in other immunological characters. Besides these toxic properties, which appear to act at a distance, there is evidence of a local action and an action on particular cells, which might be of importance in explaining the mechanism of localization of the meningococcus in the tissues and organs of the body, and these too might play a part in that almost peculiar property of the meningococcus observable in the rapidity with which it causes death in sensitized animals.

M10. Some Problems Due to Diversity Among Meningococci. SARA E. BRANHAM, National Institute of Health, United States Public Health Service, Washington.

Classifications of meningococci are discussed, and emphasis is placed upon the amount of time and labor required to "type" these microörganisms satisfactorily. Consideration is given to the factors that make "typing" of meningococci so involved: (1) the lack of strict group specificity among meningococci; (2) the consequent lack of specificity in the sera used for "typing;" (3) the tendency of the more specific or "narrow" strains to broaden antigenically until they overlap several or all groups, making it difficult (*a*) to maintain standard representative strains, and (*b*) to place in their proper groups strains that have undergone a period of laboratory maintenance.

The group relationships of meningococci found during the recent epidemics of 1928–31 are described and compared with those found in the great outbreaks of 1915–18. More than 80 per cent of recently studied strains fell into one general group corresponding to the I–III types of Gordon and Murray: whereas Group II was conspicuous during 1915–18. The significance and value of "typing" are discussed in the light of evidence presented, especially in relation to (1) choice and maintenance of standard strains; (2) choice of strains for production of therapeutic serum; and (3) routine "typing" in clinical laboratories.

M11. Dissociation of the Meningococcus Following Cultivation in Normal Rabbit's Blood. JOHN F. ENDERS, Harvard University Medical School, Boston.

The present paper describes a method by which the dissociation of recently isolated strains of meningococci may be brought about and gives an account of certain characteristics of the "rough" variant thus obtained.

After failing to induce dissociation by cultivation in broth containing 10 per cent meningococcus antiserum, an R-variant was finally produced by repeated subculture in fresh defibrinated rabbit's blood. The variant appeared after about 20 daily subcultures in that medium.

The R-variant, 24 hours after incubation at 37°C., produced colonies on 10 per cent chocolate rabbit's blood hormone agar which had granular surfaces and slightly uneven edges. By light reflected from the colony's surface, images of objects appear blurred and distorted in contrast to the sharp outlines seen on the surface of the colonies of the S-organism.

Morphologically, the R-variant differs from the S-form in being on the average somewhat larger as well as less uniform in size, and in showing a more pronounced tendency to form "tetrads" and even more complex aggregates of cohering organisms. Although repeated attempts have been made by various methods of staining, no capsule either on the R- or S-form has conclusively been demonstrated.

In peptone water containing 5 per cent rabbit's serum the S-organism grows in a pellicle which ultimately disintegrates and falls to the bottom of the tube in the form of large flakes not easily disrupted on shaking. In contrast the R-variant grows diffusely or in extremely fine granules. A delicate granular deposit is laid down which is readily dispersed on shaking.

Three out of 4 R-variants isolated from the cerebrospinal fluid failed to grow on ascitic trypagar, a medium which supported a luxuriant growth of the corresponding S-organism. One of the 4 R-variants grew on this medium although more slowly than the S-form.

In its fermentation of sugars the R-variant does not differ qualitatively from the S-organism, but the former shows a greater capacity to produce acid in the presence of maltose and glucose.

Absorption with the R-variant of an antiserum produced in rabbits by injecting the S-organism did not remove the agglutinin for the latter, but absorption with the S-organism of an anti-R rabbit serum removed the agglutinins for both R and S forms.

These data would seem to imply that the antigenic structure of the R-form is less complex than that of the S-organism.

After repeated subculture in fresh rabbit's blood, the R-variant exhibits a marked resistance to the bactericidal action of fresh normal rabbit's serum, whereas the S-organism is far less able to withstand the serum's bactericidal effect.

An attempt has been made to compare the virulence in mice of the R and S-forms. Although the results of such experiments in the case of the meningococcus can never be unreservedly accepted, it has been repeatedly shown that the minimum lethal dose of the R-organism is at least 12 times greater than that of the S-form.

M12. Further Studies on Meningococcus Toxin. M. A. WILSON (by invitation) AND C. V. RILEY, Neurological Institute of New York.

This is a progress report on our study of certain meningococcus preparations. The toxicity of our defatted antigen was compared with the toxicity of three other meningococcus preparations, namely, live culture, Shwartzman's "toxin" and Murray's "endotoxin."

The mouse protection test with the standard meningococcus horse serum and with Shwartzman's antitoxic meningococcus horse serum was the method used in these experiments to measure the toxicity of the preparations.

The four preparations were all toxic for mice. The standard therapeutic meningococcus horse serum completely protected the mouse against the defatted coccus in two-thirds of the tests. It completely protected against live cultures in all cases. It protected against Shwartzman's toxin and Murray's endotoxin. The normal horse serum gave no protection against any of the preparations. The results with Shwartzman's horse serum are not yet available.

M13. Variations in Neutralizability of Meningococcus Toxins, as Measured by the Phenomenon of Local Skin Reactivity. GREGORY SHWARTZMAN, Laboratories of the Mt. Sinai Hospital, New York.

In a previous communication it was reported that the neutralizability of meningococcus reacting factors underwent a considerable change if the strain from which the factors were derived was passed through mice or cultured for a number of generations in media containing immune antimeningococcus serum.[1]

In the work reported here, toxins from a number of strains of various serological groups kindly supplied by Drs. A. Wadsworth and S. Branham, as well as toxins derived from some of these strains cultured in media containing human blood for 8 to 10 generations were tested against mixture of a given antimeningococcus horse serum with the auxiliary antibody.[2] The following titers of complete neutralization were obtained:

Toxins from "stock" strains: Group I: "440" = 50 units, "397" = 90 units, "401" = 120 units, "130" = 55 units, "178" = 120 units. Group II: "383" = 150 units. Group III: "44B" = 110 units, "396" = 165 units, "101" = 75 units.

Toxins from "human blood" strains: Group I: "440" = 20 units, "130" = less than 10 units. Group II: "383" = 35 units. Group III: "44B" = 60 units, "396" = less than 50 units.

As is seen, there is a considerable difference in neutralizability of toxins obtained from various strains of the same serological groups. Similar observations with *B. typhosus*[3] suggest that this is most likely due to the fact that strains of lower neutralizability have a more complex antigen. It seems important, therefore, that these strains be included in the immunizing material used for horses.

It is also evident that there is produced a marked lowering in neutralizability of toxins derived from strains passed through human blood. In view of this, it is suggested that attempts be made to obtain for therapeutic purposes sera which will neutralize satisfactorily toxins of "human blood" strains.

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[1] Shwartzman, G.: Jour. Inf. Dis., 1931, **48**, 339.

[2] Shwartzman, G.: Jour. Exp. Med., November, 1931.

[3] Shwartzman, G.: Jour. Exp. Med., 1930, **52**, 781.

M14. Meningococcus Antitoxin: Prophylactic and Therapeutic Tests on Monkeys and Other Laboratory Animals. N. S. Ferry, Research Laboratories, Parke, Davis & Company, Detroit, Mich.

In a paper recently published in the Journal of Immunology (1931, vol. 21, no. 4), Ferry, Norton and Steele showed that four- to six-day bouillon filtrates of the four Gordon types of meningococcus possess properties characteristic of a soluble toxin and that these filtrates stimulate in animals specific serums antitoxic in character.

In the present experiments an attempt has been made to demonstrate on monkeys and guinea pigs the antitoxic properties of such a serum prepared by hyperimmunizing the horse by alternate injections of the four toxins. Acute meningitis was first produced in monkeys by intraspinal injections of virulent cultures of the meningococcus. After typical severe symptoms of infection appeared, usually in about five to seven hours, the experimental meningococcus antitoxin was injected intraperitoneally. Of the sick monkeys so treated eighty-one per cent recovered.

Of the control monkeys, similarly injected with culture alone and exhibiting similar severe symptoms, none recovered.

Several of these serum treated monkeys which recovered were later intraspinally reinjected each with a massive fatal dose of culture. The interval between the primary injection and the reinjection with the massive dose ranged from five to twelve weeks. All recovered.

Of the serum treated monkeys injected with the massive dose of virulent culture, with only one week between the initial injection and massive dose, none recovered.

In the experiments on guinea pigs both the culture and serum were always injected intraperitoneally.

The results of a large number of experiments, well controlled with normal horse serum and serum from horses immunized in the usual manner with live organism, showed that the experimental antitoxin would protect against fatal issue when injected before, simultaneously and at least three hours after inoculation with fatal dose of culture.

M15. A Poisonous Substance Obtained from Meningococcus. WILBUR G. MALCOLM, Massachusetts Department of Public Health, Antitoxin and Vaccine Laboratory, Boston.

Poison, similar to that obtained by Gordon and others, can be obtained from fresh, moist, young cultures of meningococci grown on a solid medium. Such cultures are rapidly and thoroughly washed in chilled, distilled water to remove metabolic products, the cells are ruptured by means of osmotic pressure, then acetic acid added to a pH of 4.0 to precipitate protein. This precipitate and the cell detritus are then removed by centrifugalization and discarded. This method of preparation minimizes the possibility of the formation of toxic autolytic products.

Such extracts prove to be toxic when injected into mice, guinea pigs, and rabbits; an extract representing 1 mgm. of meningococcus kills a 20 gram mouse within 24 hours. The product is stable, and permits accurate determinations of its lethal dose. It is heat resistant; it is comparatively low in nitrogen content; it is inactivated by strong alkali but can be reactivated by acetic acid. Its toxicity is destroyed by hydrochloric acid.

When such an extract is injected into rabbits intradermally, a local reaction takes place, characterized by marked induration and erythema which goes on to necrosis. When the material is injected subcutaneously a severe local reaction is produced, but after repeated subcutaneous injections these reactions decrease in severity, pointing to the production of tolerance or immunity.

This poisonous substance appears to be antigenic in rabbits since after its repeated subcutaneous injection, the serum of such rabbits neutralizes the poison, as shown by the injection into mice of poison-immune serum mixtures.

M16. Further Studies on the Meningococcus with Reference to the Shwartzman Reaction. H. M. POWELL, Lilly Research Laboratories, Indianapolis, Indiana.

The preparation of a toxic principle from meningococci as described by Shwartzman has been verified, and the action of several batches of antiserum upon this substance has been studied.

In the absence of pertinent information as to the exact nature of this reactive principle and the mechanism of its reaction with antiserum, it appears that some sera inactivate it better than others. The clinical use of antisera more effective in this respect appears to be coupled with a decrease in the toxicity of the patient during serum treatment.

M17. The Specific Treatment of Influenzal Meningitis. LEROY D. FOTHERGILL, JOYCE WRIGHT AND HUGH K. WARD (by invitation), Harvard University Medical School and Infants' and Children's Hospital, Boston.

During recent years, cases of acute purulent meningitis due to *B. influenzae*, pneumococcus and streptococcus have been as frequent as cases of meningococcus meningitis in children admitted to the Children's Hospital, Boston. Actually, the influenza bacillus has been found in some 25 per cent of all cases of acute purulent meningitis. It is a disease of infancy and very early childhood, and is almost invariably fatal.

Specific therapy in meningococcus meningitis having proved so successful, it was thought worth while to attempt a similar treatment in influenzal meningitis. Rivers reported some years ago that the strains of *B. influenzae* found in meningitis were homologous and this has been our own experience. The homogeneity of the organisms of course simplified the problem of producing an antiserum for use in these cases.

A horse was accordingly immunized with formalinized cultures of smooth, virulent organisms. The antiserum was at first tested by agglutination, but later a bactericidal test was used, as it could be shown that the presence of agglutinins in the antiserum did not necessarily connote any bactericidal activity. The precipitins in the antiserum, on the other hand, appear to parallel accurately the bactericidins, and these two antibodies are presumably identical. The precipitinogen employed is a filtrate of a broth culture of the virulent organism.

Kolmer has pointed out that the cerebrospinal fluid in these cases is lacking in complement, and we have never found the slightest trace of this substance in any of the cases. In meningococcus meningitis, on the other hand, complement has been present in all the specimens of cerebrospinal fluid that we have tested. In influenzal meningitis the spinal fluid has often a high cell count, but *in vitro* experiments show that leucocytes and antiserum have only a slight bactericidal action on the organisms in the absence of complement. It thus seemed advisable to inject complement along with the antiserum, as indeed has been advocated by Kolmer.

We have now treated some 12 cases with antiserum and complement, using fresh, human serum as the source of complement. The injections were made alternately by the lumbar and ventricular route, and where the blood culture was positive, antiserum was injected intravenously. In the majority of cases, the cerebrospinal fluid has been sterilized temporarily for varying periods, but never permanently except in one case, and all the children ultimately died with this one exception. In this case, reported to us by Dr. A. Kuttner of Johns Hopkins Hospital, the organisms disappeared after the first treatment with antiserum and complement, and the child recovered. Such autopsies as have been obtained in the cases where the cerebrospinal fluid was temporarily sterilized have shown localized, walled-off abscesses in the subarachnoid space, particularly at the base of the brain and in the frontal areas of the cerebral cortex. At the present time, it is impossible to say whether the unsatisfactory results are due to the mechanical barrier of the abscess walls, or to some inherent defect in the application or content of the antiserum and complement. The fact that the free cerebrospinal fluid can be sterilized temporarily in most of the cases is perhaps somewhat in favor of the mechanical obstruction hypothesis.

Poliomyelitis, Typhus and Miscellaneous

M18. The 1931 Strain of Poliomyelitis Virus. SIMON FLEXNER (by invitation), Rockefeller Institute, New York.

M19. The Epidemiology of Poliomyelitis. W. LLOYD AYCOCK, Harvard Medical School, Boston.

The efficacy of serum treatment of poliomyelitis is still in question; a more abundant supply of immune (convalescent or normal human) or hyperimmune artificial serum would not, on account of practical difficulties in early diagnosis and treatment, offer an adequate means of combating the disease. Other defenses must depend on a knowledge of the epidemiology of the disease. In view of evidence that the widespread dissemination of the virus is a function of ordinary and probably unavoidable human contact bacteriological control of the disease is unlikely. Since the preponderant majority of individuals exposed to the virus do not develop the paralytic disease, on any basis of selection which present knowledge permits, active or passive immunization would have such a low percentage efficacy that the feasibility of immunological control of the disease is doubtful. Epidemiological and clinical observations raise questions as to whether the physiologic fault which renders a relatively small proportion of individuals susceptible to the paralytic disease may be hereditary or environmental, endocrinological or nutritional, seasonal or climatic, or, temporary or permanent. Until the nature of host susceptibility is determined with a view either to the selection of susceptible individuals for artificial immunization or the autarceological control of the disease, in the individual or *en masse*, all the possibilities for combating the disease will not have been exhausted.

M20. The Production of Anti-Anterior Poliomyelitis Serum in the Horse and the Use of this and Convalescent Serum in Prevention and Treatment. WILLIAM H. PARK, Bureau of Laboratories, Department of Health, New York City.

An unfiltered emulsion of the cords of monkeys dying of poliomyelitis is an efficient antigen for the preparation of an antivirucidal serum against poliomyelitis. The injections are given subcutaneously and intracutaneously in repeated and increasing doses. The effect of the horse serum when given in children intraspinally is deleterious as it excites an aseptic meningitis. The results in New York indicate that convalescent serum or serum from persons who have antibody in their blood without having had the disease is utilized too late when we come to treat preparalytic cases. So far as immunization is concerned, there have been several instances of mild anterior poliomyelitis developing after the use of the convalescent serum as an immunizing agent.

M21. The Antibody Titer of So-Called "Normal" Adult Serum Compared to that of Convalescent Poliomyelitic Serum.

MAURICE BRODIE, Department of Bacteriology, McGill University, Montreal, Canada.

Many observers have shown that the blood of individuals with no history of clinical poliomyelitis, or even of exposure, may possess antibody which neutralizes the virus. In the present work, therefore, it was proposed (1) to determine the antibody content in a series of "normal" adults who gave no history of clinical poliomyelitis, and (2) to compare the antibody titer of a number of these individuals with that contained in human convalescent serum. The method used was to compare the neutralizing power of the serum being tested against a known amount of virus estimated in minimal lethal doses.

Four pooled convalescent sera were tested against 80 m.l.d. of poliomyelitis virus. The strength of the standard emulsion remained constant throughout the experiment. The results were constant for a serum whether or not it was phenolated. The titrations showed that pooled sera from various groups differed considerably in their strength. A pooled serum 2 years old still showed considerable neutralizing power.

Out of 28 so-called "normal" urban adults, 22 showed approximately one-half the antibody content of the average convalescent serum. On further titration, more than half of these had neutralizing power equal to that of convalescent serum. The incidence and degree of demonstrable immune bodies were not influenced by any history of definite contact.

(This research was made possible by a grant from the Banting Research Foundation.)

M22. Typhus Fever Studies.

HANS ZINSSER AND M. RUIZ CASTANEDA (by invitation), Harvard Medical School, Boston.

This paper considers the relationships and differences between guinea pig infection with the viruses of the following diseases:

(1) European and North African typhus, (2) Mexican and the North American type of Maxcy, (3) Brill's disease, (4) the Malayan disease, (5) the Brazilian Sao Paulo disease. Transitional conditions between these diseases and Rocky Mountain spotted fever are discussed, particularly in their relation to the eastern type of the latter disease described by Rumreich, Dyer and Badger.

The epidemiology of Mexican and American typhus fever, especially in regard to rat transmission, propagation of the disease from rat to rat, from rat to man and from man to man is discussed, particularly in regard to the rat studies, rat louse studies and the work on fleas, bed bugs and ticks carried out by the authors. The conclusion of this part of the paper is that we are dealing with a closely related group of diseases in which guinea pig study shows transitional stages, and that these diseases are originally rodent infections which are kept alive in rodents by insects and in the transmission of which from rodents to man a considerable number of insects probably play a rôle, with emphasis on individual insects in different regions of the world.

Confirmations of crucial studies of the etiology of the Rickettsia are mentioned. Negative cultivation studies with various media, including Kendall's and chick embryo media, are discussed. Experiments with the implantation in guinea pigs of glass capsules filled with Rickettsia are discussed in relation to the biology of Rickettsia. All the authors' cultivation studies of a number of years indicate that the Rickettsia cannot multiply except inside of living cells and only within certain specialized cell groups of endothelial origin. No evidence whatever has been found in the work of the writers that the Rickettsia have any dissociation relationship whatever with any form of Proteus.

Improvements and difficulties in vaccine production with formalinized Rickettsia are outlined. External temperature, age of the rats used and careful attention to dosage of benzol are the most important points in vaccine production by the method of the authors.

There is a discussion of the probable origin of isolated typhus cases encountered in northern cities like Boston.

M23. The Typhus-Rocky Mountain Spotted Fever Group in the United States. A. Rumreich, National Institute of Health, Washington.

A mild form of typhus fever is endemic in the United States. This disease is not louse-borne. Epidemiological evidence has indicated the existence of a rodent reservoir of the disease, with transmission from rat to man by means of the rat flea. The virus has been recovered from rat fleas obtained at typhus foci. Experimental transmission from rat to rat by means of the rat flea has been effected.

By epidemiologic and clinical studies, a type of spotted fever hitherto confused with typhus fever has been recognized and differentiated. The immunologic identity of the virus of this disease with that of Rocky Mountain spotted fever has been established. Experimental transmission of the disease in guinea pigs by means of the American dog tick, incriminated as the vector by epidemiologic evidence, has been carried out.

M24. The Stability of the Toxic Principle in Diphtheria Toxin. William Edward Bunney, Michigan Department of Health, Lansing.

If 0.4 per cent phenol is added to diphtheria toxin diluted to Schick strength in saline solution, the toxicity rapidly disappears at room temperature. The presence of 2 per cent Difco proteose peptone has been previously shown to slow down this drastic action of phenol so that Schick-strength diphtheria toxin will keep for months at room temperature. In an attempt to determine the fraction of the Difco proteose peptone which might be responsible for this protective action, it was observed that Witte peptone was more efficacious, concentration for concentration, than Difco proteose peptone. It was found that 0.25 per cent Witte peptone gave sufficient stabilization to be used as a Schick diluent and had the added advantage over two per cent Difco proteose peptone in that the Schick reactions from injections of the 0.25 per cent Witte Schick preparations were identical with those resulting from injections of the usual saline-diluted Schick material, regardless of the age or previous history of immunization of the people injected. A further study of the question has been made by investigating the relative stabilizing effect of other peptone and nitrogenous-compound solutions with different molecular sizes, ranging from that of simple amino acids to the largest split products of protein digestion. Some study of the effect of merthiolate on Schick strength diphtheria toxin has also been made.

M25. Dissociation of the Specific Protein Precipitate of Antipneumococcus Horse Serum, and a Comparison with a Protein Isolated by Chemical Means from this Immune Serum. LLOYD D. FELTON, Harvard Medical School, Boston.

It has been found possible to separate the active protein of the total water-insoluble fraction of antipneumococcus horse serum from the inactive protein by means of metal salts. The metal-protein solution is completely precipitated by pneumococcus soluble specific substance. The water-insoluble protein thus prepared is found to be similar to that obtained by dissociation of the protein from the specific precipitate made by adding soluble specific substance to antipneumococcus serum. The proteins prepared by the two methods are water-insoluble, both have isoelectric points to the alkaline side of euglobulin, both are soluble in 30 per cent saturation of ammonium sulfate and insoluble in 44 per cent saturation, and both alike are protective and otherwise immunologically active.

M26. Antibody Formation in Animals Treated Intracutaneously and Subcutaneously with Killed Cultures of Staphylococcus aureus. RACHEL E. HOFFSTADT AND PHILIP ALMADEN, Department of Bacteriology, University of Washington, Seattle.

Rabbits were treated with heat-killed cultures of *Staphylococcus aureus* intracutaneously, subcutaneously and with a combination of intracutaneous and subcutaneous treatment. Agglutinins and complement fixations were tested for at weekly intervals for three months. Total white blood counts and differential blood counts were made over a period of three months for each group. At the end of that time half of the animals were given a lethal dose of the organisms and half a sublethal dose. In the last half, hourly blood cultures over a period of 24 hours were made and the disappearance of the organisms from the blood stream and the dissociation of the organism were studied.

M27. Quantitative Determination of the Bacteremia in Pneumococcus Pneumonia and its Value as a Prognostic Indicator. K. R. KOCH, Institute of Pathology, Western Pennsylvania Hospital, Pittsburgh.

The incidence, degree and trend of bacterial blood stream invasion were determined by consecutive blood cultures in a series of 139 patients not treated with antipneumococcus serum. Glycerophosphate media showed characteristic changes within 12 to 14 hours, if the degree of bacteremia was one or more organisms per cubic centimeter of blood.

None of the patients who had an increasing or stationary degree of bacteremia, survived the infection. The increasing bacteremia predicted the fatal outcome 1 to 10 days preceding death.

Of 12 patients, in whom the bacteremia decreased and disappeared, 10 recovered.

In none of the patients who overcame the infection did the maximum degree of bacterial invasion of the blood stream exceed 30 organisms per cubic centimeter of blood. The relations of the trend of the bacteremia to the stage of the disease when the bacteremia was first demonstrated, the pneumococcus types, virulence of the organisms and resistance of the patient are discussed.

M28. (*a*) *The Effect of Fever Temperatures on Treponema pallidum.* R. A. BOAK AND C. M. CARPENTER. (*b*) *The Effect of Fever Temperatures on N. gonorrheae.* R. A. BOAK, C. M. CARPENTER AND L. A. MUCCI, Rochester School of Medicine and Dentistry, Rochester, New York.

(*a*) Extracts of rabbit testes infected with *Treponema pallidum* were placed in vials sealed and immersed in water baths at temperatures ranging from 37.5° to 42°C. The intratesticular inoculation of extracts so treated demonstrated the lethal effect of certain time temperature combinations. Rabbits infected with *Tr. pallidum* and later given similar fevers by short radio waves failed to produce syphilis when their testicular extracts were injected into other rabbits.

(*b*) Forty-eight hour cultures of 15 strains of *N. gonorrheae* were placed in vials, sealed and subjected to water bath temperatures ranging from 37.5° to 42°C. Such temperatures as 40°, 41°, 41.5°, and 42°C. destroyed this organism as determined by culture methods.

M29. The Influence of Radium Treatment Upon the Bacterial Flora of the Female Genitalia. E. PRIBRAM AND H. SCHMITZ, Loyola University School of Medicine, Chicago.

The influence of radium treatment upon the bacteria of the cervical secretion was studied by bacteriological examinations of the cervical flora of untreated and treated patients at different times during and after treatment.

The essential results were: (1) Extensive destruction of the bacterial flora and considerable diminution of micro-organisms for a period of half a year and longer after a treatment of 14 days. (2) Preservation of leucocytes (polymorphs, lymphocytes, monocytes) in a resting stage with absence of pseudopodia and phagocytosis.

M30. A Note of the Variability of the Germicidal Action of Normal Human Skin. JUSTINA H. HILL AND EDWIN C. WHITE, James Buchanan Brady Urological Institute, Johns Hopkins Hospital, Baltimore.

Determination of possible bacteriostatic, bactericidal or bacteriolytic action of normal human skin was the object of these experiments.

The method consisted essentially of bringing normal human skin in contact with agar plates, either soon after their inoculation, or after twenty-four hours of incubation. A variety of different organisms were used, including anthrax spores, a non-pathogenic spore-former, *Staphylococcus aureus*, Proteus, *B. pyocyaneus*, and different strains of Escherichia and of Aerobacter. Tests were made on a number of individuals on different days.

The experiments showed the following variations:

1. The skin may have powerful bacteriostatic, bactericidal or even lytic action.
2. The skin may be entirely lacking in any action on bacteria.
3. The variations occur in at least three ways, (*a*) with the test organism, (*b*) with the individual tested, and (*c*) with the same individual on different days.

M31. Bacteriologic Testing of Catgut Sutures. Ralph Oakley Clock, New York City.

Because of the large percentage of contaminated commercial catgut sutures reported in various countries by competent bacteriologists, the careful bacteriologic testing of such sutures by an approved method and with adequate controls is of paramount importance to bacteriologists as well as to the surgical profession.

During the past fourteen months, the standard test proposed by Meleney and Chatfield was used with three additional and essential controls, which are described and recommended, for determining the sterility of many thousand lots of surgical catgut sutures. In applying this test to copperized catgut sutures, the results indicated sterility; but the neutralizing solution of sodium thiosulphate and sodium carbonate for removing chemical compounds, which is embodied in the standard test, was found to be ineffective for leaching out copper salts.

After many experiments, a neutralizing solution of five per cent ammonium chloride with one-half per cent ammonium hydroxide was devised, and was found to effectively remove the copper salts from catgut sutures. The practical value of this neutralizing fluid for use with the standard bacteriologic test was first determined by artificially infecting catgut ribbons, which were then impregnated with copper salts and made into finished sutures. After removing the copper salts aseptically with this special neutralizing solution, the sutures were put through the standard bacteriologic test and the results showed bacterial growth.

Copperized catgut sutures of three American firms, as well as those of one German manufacturer, were next treated aseptically with this neutralizing solution of ammonium chloride and ammonium hydroxide; and then they were subjected to the standard bacteriologic test of Meleney and Chatfield. The results showed the presence of living bacteria in seven out of twenty-four (29 per cent) of the German manufacturer's sutures, and in twenty-four out of eighty-four (28½ per cent) of the sutures of the three American firms.

M32. The Incidence of L. acidophilus in Adults. N. KOPELOFF, N. BLACKMAN AND B. MCGINN, Department of Bacteriology, The Psychiatric Institute, New York.

The incidence of *L. acidophilus* was determined in 208 subjects using casein digest agar for plating fecal specimens.

Of 187 patients with mental disease 62 per cent harbored *L. acidophilus.* The incidence of this lactobacillus was slightly higher in the psychogenic than in the somatogenic syndromes. Of 21 non-psychotic adults one-half the number harbored *L. acidophilus.* Shera's conclusion that there is an acidophilus deficiency in mental patients is consequently invalid from the data at hand.

L. acidophilus was found in the same proportion of constipated patients as in non-constipated patients. Within the limitations of the material there is no correlation between constipation and the absence of *L. acidophilus* in fecal specimens.

M33. The Effect of Alum on Horses Used for the Production of Diphtheria Antitoxin. GEORGE F. LEONARD AND JAMES R. VARLEY, Biological Laboratories, E. R. Squibb & Sons, New Brunswick, N. J.

Three groups of horses were immunized for the production of diphtheria antitoxin. Group 1 received toxoid containing 0.5 per cent potassium alum, followed by diphtheria toxin containing alum. Group 2 received diphtheria toxoid, followed by diphtheria toxin,—both without alum. Group 3 received diphtheria toxin-antitoxin, followed by diphtheria toxin,—both without alum. As a result of the marked intolerance of the horses of Group 1 toward alum antigens, they received less than three-tenths of the antigen dosage given to the horses of Group 2.

Based on the relative antigen doses and the subsequent antitoxin titers, the antigenic effectiveness of immunization with alum-treated toxoid and toxin was about three times as great as that of toxoid and toxin without addition of alum.

The cumulative toxic effect was observed in the horses of Group 1 after *repeated* doses of alum combined with diphtheria antigens. During a period of several weeks after injections containing alum had been discontinued, most of the horses in this group gradually declined and two-thirds of them died with symptoms of alum poisoning.

The disastrous late after-effects upon horses of *repeated* injections of alum along with diphtheria antigens completely overshadow any increase in immunizing power attributable to the addition of the alum.

M34. A Study of the Granular and Floccular Types of Agglutination of B. typhosus. RUTH GILBERT, MARION B. COLEMAN AND ALICE B. LAVIANO, Division of Laboratories and Research, New York State Department of Health, Albany.

Recent work indicates that the demonstration of granular and floccular agglutinative properties of sera may furnish information of more diagnostic and possibly prognostic significance than can be secured from the results of a single test with a living culture of *B. typhosus.* Furthermore, owing to the tendency of cultures of *B. typhosus* to vary in their agglutinative properties under conditions of artificial cultivation, the use of satisfactory suspensions of killed typhoid bacilli in agglutination tests should lead to more reliable results.

In addition to the routine agglutination test with *B. typhosus,* two antigens, one treated with formalin to demonstrate floccular agglutinative properties and the other with alcohol to demonstrate those of the granular type, were used in the examination of a series of specimens submitted for the agglutination test for evidence of typhoid or undulant fever or for the complement-fixation test for syphilis. While the results of the study are too limited to warrant definite conclusions, they indicate that the employment of two such antigens will furnish at least as much information as can be secured with tests with living culture and will probably provide more data of diagnostic value.

M35. Agglutinin Absorption Studies on the Genus Brucella.

WAYNE N. PLASTRIDGE, Storrs Agricultural Experiment Station, Conn.

A study of the serological characteristics of 142 strains of Brucella of human, bovine, porcine and caprine origin has been made for the purpose of comparing the results of the agglutinin-absorption test with the ability to utilize dextrose and to grow on liver-infusion agar containing the following dyes: methyl violet (1:100,000), basic fuchsin (1:50,000) and thionin (1:50,000), respectively. Antiserums against *Br. abortus* (bovine type), *Br. abortus* (porcine type) and two strains of *Br. melitensis* (M39 and M64) which appeared to differ serologically, were prepared and the absorbing dose with the homologous antigen determined. Approximately 1.5 times the absorbing dose was employed in the absorption tests with heterologous antigens. Absorbed serums were tested for the presence of agglutinins for both the homologous and heterologous antigens.

Of 116 strains of *Br. abortus* of human, bovine, porcine, and equine origin, 99 (85 per cent) absorbed completely agglutinins from the antiserums against *Br. abortus* (bovine and porcine types) and strain M39 of *Br. melitensis*, but failed to do so in the case of the M64 antiserums. The remaining 19 strains could be roughly grouped as follows: 12 absorbed practically to extinction the agglutinins from all of the test antiserums and 7 failed to absorb an appreciable amount of agglutinins from any of the test antiserums. No serological differences were observed between the bovine and porcine types of *Br. abortus* regardless of origin.

The 26 strains of *Br. melitensis* examined were divided into 5 groups. Group I (3 strains) absorbed completely agglutinins from *Br. abortus* antiserum, but not from the two *Br. melitensis* antiserums. Group 2 (13 strains) absorbed completely agglutinins from the M64 (*Br. melitensis*) antiserum only. Group 3 (4 strains) absorbed completely agglutinins from the M39 (*Br. melitensis*) antiserum only. Group 4 (2 strains) absorbed completely agglutinins from both M64 and M39 antiserums, but not from the *Br. abortus* antiserums. Group 5 (4 strains) failed to absorb completely agglutinins from any of the test antiserums.

The three strains placed in Group 1 appear to be serologically identical with *Br. abortus*; nevertheless, the histories of these strains, their ability to utilize dextrose, and their behavior on dye plates show that they should be classified as *Br. melitensis*.

Tests repeated from time to time on strains of *Br. abortus* and *Br. melitensis* show that under ordinary laboratory conditions of cultivation the agglutinin absorbing capacities and the agglutinability of some strains may vary from time to time. It is believed that this condition is due to the development of cells in the stock culture which possess mucoid characteristics, and that this tendency to vary serologically may help to explain the lack of agreement in the results reported by various investigators.

In general, the results obtained confirm the opinion expressed by Burnet, namely that, while it is possible to classify a given strain as *Br. melitensis*, providing it does not possess a high degree of the para or mucoid characteristics, it is impossible to identify strains of *Br. abortus* by means of the agglutinin absorption test with any degree of certainty.

M36. Brucella abortus in Human Tonsils. L. J. Poelma and E. M. Pickens, Department of Bacteriology and Pathology, University of Maryland, College Park.

To study the rôle of *Br. abortus* in tonsillar infections, 117 pairs of tonsils were cultured from patients, living in rural districts, who presumably drank raw milk. The cultures were incubated at 37°C. in an atmosphere containing about 10 per cent CO_2 for 4 or 5 days. These cultures showed various streptococci and staphylococci present; but in no instance was *Br. abortus* isolated directly from the tonsils.

Guinea pigs were injected with 1 cc. of an emulsion of a whole tonsil in sterile physiological salt solution, two animals being used for each pair of tonsils. Only 116 pairs of tonsils were used for injection, the other pair being too badly decomposed upon arrival at the laboratory. After 6 or 7 weeks, blood samples were obtained from the animals for the agglutination test, after which the guinea pigs were killed and cultures were made from the livers, spleens, and certain lymph glands. Four animals representing two pairs of tonsils gave positive agglutination tests, and the organisms were recovered from them. Three of these had enlarged spleens, and the fourth showed no lesions of any kind.

M37. Filterable Virus Carriers. CHARLES S. GIBBS, Massachusetts Agricultural Experiment Station, Amherst.

Filterable virus carriers are suspected in rinderpest and hog cholera for a long time. In these diseases the virus may be located in ulcers persisting in the alimentary tract after all acute symptoms have subsided. Recently, filterable virus carriers have been found in infectious trachitis of fowls. In this disease the virus persists for indefinite periods in the tracheas of a small number of birds recovered from the acute stages and apparently in good health. The discovery of carriers in the filterable virus diseases mentioned above indicates the possibility of similar carriers in other filterable virus diseases.

M38. The Californian Strain of Myxoma Virus. JOHN F. KESSEL AND ROY T. FISK, School of Medicine, University of Southern California. Los Angeles. (Read by title.)

During the summers of 1930 and 1931 several outbreaks of infectious myxomatosis have been encountered in rabbitries in Southern California. A comparison of the symptoms and pathology produced by strains of virus recovered from these outbreaks with a South American strain of myxoma virus provided by Dr. Rivers of the Rockefeller Institute indicates that the two strains produce essentially the same conditions. The only possible difference is one of virulence since the incubation period of the Californian strain is slightly longer than that of the South American strain and further, among 162 animals inoculated with the

Californian strain of virus without prophylactic measures, two have recovered.

Immunologic studies with chemically treated virus, heat-treated virus, and with virus neutralized with immune serum have shown that the first has completely protected animals in 10.2 per cent of the cases and prolonged the length of life for a period of more than 10 days in 15.4 per cent of the cases, 39 vaccinated animals and 29 unvaccinated control animals being used in the experiment. Heat-treated virus has produced no resistance and virus neutralized with immune serum was non-pathogenic to 4 of 8 animals. These 4 animals, however, were non-resistant to subsequent inoculation with untreated virus. Rabbits immunized against and recovered from the Californian strain of virus were also immune to inoculation with the South American strain of virus.

TWO RAPID METHODS FOR DISTINGUISHING BETWEEN ESCHERICHIA COLI AND AEROBACTER AEROGENES

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The methods for distinguishing between *Escherichia coli* and *Aerobacter aerogenes* which are given in the "Standard Methods for Water Analysis" (1925) are subject to two serious criticisms from the standpoint of the practical water laboratory. The most serious objection is that five days are required for the tests after the organisms have been obtained in pure culture. The second objection is that very specialized media are required.

The methods to be presented in this paper compare favorably with the "Standard Methods" in reliability, require only the ordinary media, and afford a tremendous saving in time.

MODIFIED VOGES-PROSKAUER TEST

The procedure for the Voges-Proskauer test outlined in the "Standard Methods" calls for incubating cultures in the methyl-red medium (synthetic or proteose-peptone) for four days at 37°, then adding 5 cc. of 10 per cent KOH to 5 cc. of culture and incubating for an additional twenty-four hours before reading. Werkman (1930) found that if two drops of a 1 per cent solution of $FeCl_3$ are added to the culture before the KOH, the tubes can be read in about twenty minutes. He also claims that the copper coloration of the positive test is more easily observed than the eosin coloration of the older test. He does not state what medium he used, but recommends an incubation period of four days.

Werkman's method gave such strong readings in the hands of the present authors, that the possibility of shortening the incubation period was investigated. It was found at the very outset that the laboratory strain of *A. aerogenes* gave a strongly positive test after twenty-four hours incubation at 37° in standard glucose broth (5 grams glucose, 5 grams bacto-peptone, and 3 grams bacto-beef extract in 1000 cc. of water). Forty-five cultures of the coli-aerogenes group were isolated from soil and water and tested by both the $FeCl_3$ method on twenty-four hour glucose broth cultures and the standard procedure using four days' incubation in the synthetic medium. The results checked exactly.

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METHYLENE BLUE REDUCTION TEST

Since other studies of the senior author had indicated that there was a difference in the oxidation-reduction potentials between *E. coli* and *A. aerogenes* under certain conditions, an attempt was made to distinguish between the two organisms upon the basis of dye reduction. It was found that when one drop of a saturated aqueous solution of methylene blue was added to a twenty-four hour culture of *A. aerogenes* in a lactose-broth fermentation tube, the methylene blue was reduced within a period of a few minutes. In the case of *E. coli*, no visible reduction took place within a period of several hours.

TABLE 1

NUMBER OF CULTURES	STANDARD METHODS		24-HOUR INCUBATION	
	Methyl-red test	Voges-Proskauer test	Methylene-blue test	Voges-Proskauer test
21	−	+	+	+
19	+	−	−	−
4	?	−	−	−
1	+	−	?	−

The 45 cultures isolated for the above study were tested in the following manner: Lactose-broth fermentation tubes were inoculated and incubated at 37° for twenty-four hours. One drop of a saturated aqueous solution of methylene blue was added, the tubes were shaken, permitted to stand for one hour, and then observed for dye reduction. The results of this test, as well as those from the "Standard Methods" Voges-Proskauer and methyl-red tests (synthetic medium), and the proposed modification of the Voges-Proskauer test are given in table 1. It is seen from table 1 that the methylene blue reduction test agrees with the Voges-Proskauer test in 44 out of 45 cases but that there are several inconsistencies with regard to the methyl-red test.

The lactose fermentation-tube used in the "completed test" for the coli-aerogenes group can also be used for the methylene blue reduction test. Thus, the time required for distinguishing between *E. coli* and *A. aerogenes* is reduced to one hour and no specialized media are required.

It is to be emphasized that the difference between the two organisms in ability to reduce methylene blue exists only under the conditions of the test. A twenty-four-hour nutrient-broth culture of *E. coli* reduces methylene blue very rapidly. If the methylene blue is added to the lactose broth at the time of inoculation, it becomes reduced after several hours incubation and may or may not become oxidized again within the twenty-four-hour incubation period.

Potentiometric measurements were made upon one strain of *E. coli* and one strain of *A. aerogenes* in order to determine what parallelism existed between the potentials developed by the cultures and their ability to reduce methylene blue.

In making the electrical measurements, 10 cc. of the culture were placed in a culture tube of 16 mm. diameter. No precautions were used to exclude air. The electrode, a bright platinum wire, was placed in the bottom of the tube. A saturated KCl calomel half-cell was used as a reference. The reference half-cell was connected with the culture tube by means of a saturated KCl bridge. Both the calomel half-cell and the electrode vessel were immersed in a water bath held at 37° ± 0.5°. All measurements were made by means of a Leeds and Northrup Type K Potentiometer using a Weston cell as a standard. The results are expressed in the terms of the potential difference in volts from a normal hydrogen electrode (E_h). The value of the saturated KCl calomel half-cell at 37° was taken to be +0.236.

Twenty-four hour lactose-broth cultures of the strain of *E. coli* used gave unsteady E_h values of +0.246 to +0.256 volts. The pH of this culture was 4.4. According to Clark, Cohen, and Gibbs (1925), the mid-point of the methylene blue-methylene white system at this pH is +0.143. Thus it is seen that this culture of *E. coli* is definitely oxidizing to the methylene blue-methylene white system.

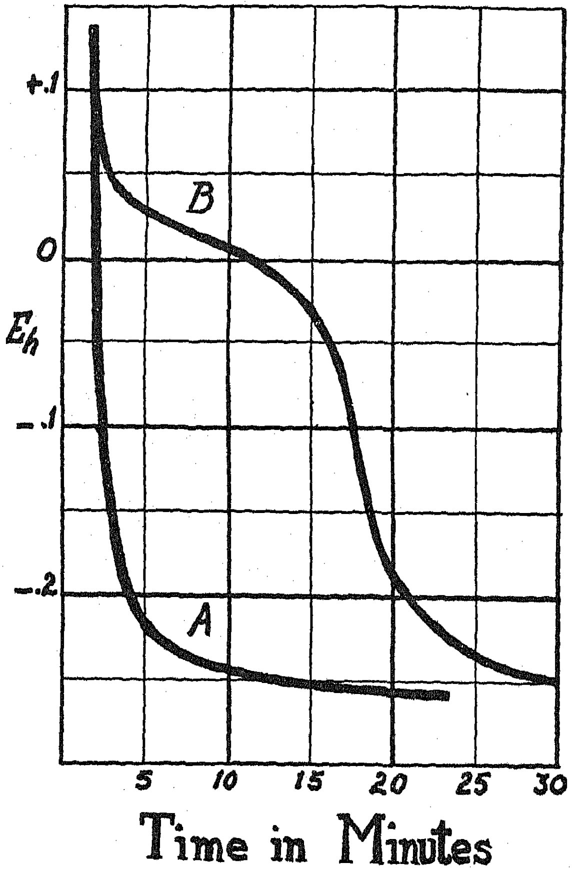

FIG. 1. TIME-POTENTIAL CURVES FOR A TWENTY-FOUR-HOUR LACTOSE BROTH CULTURE OF *A. aerogenes*

Curve *A* is for the culture alone. Curve *B* is for the culture plus methylene blue.

The twenty-four-hour lactose-broth culture of *A. aerogenes* gave an E_h value of -0.252 volts. The pH was 6.4. Using Clark's results again, the mid-point of the methylene blue-methylene white system at this pH is approximately $+0.03$. It is apparent that this culture is decidedly reducing to the methylene blue-methylene white system. The fact that the culture does reduce methylene blue in the test shows that it has sufficient capacity to reduce the amounts used.

The reduction of methylene blue by the lactose-broth culture of *A. aerogenes* was followed potentiometrically in the following manner: One drop of a saturated aqueous solution of methylene blue was added to 10 cc. of the culture and the tube was shaken vigorously for one minute. The appropriate connections were made and a reading taken as soon as possible. Subsequent readings were made at frequent intervals until sometime after the methylene blue was reduced. The process was timed by means of a stop-watch which was started the instant the shaking of the tube was stopped. The results are shown graphically in figure 1. The time-potential curve for the culture without the methylene blue is given for comparison.

From figure 1, it is seen that when the culture is shaken with air, the potential becomes much more positive (oxidizing) but that when the shaking is stopped, the potential rapidly returns to its negative limit. If methylene blue is present, the culture passes rapidly into the methylene blue-methylene white system, progressively reduces the methylene blue, and then passes smoothly out of the system. The extent to which the curve is offset by the methylene blue represents the amount of work necessary to reduce the methylene blue to methylene white, or, in other words, the "poising" effect of the methylene blue.

In view of the fact that a bacterial culture is such a complex system and that only commercial methylene blue was used in this study, the agreement of the results with those of Clark, Cohen, and Gibbs (1925) for purified methylene blue in aqueous solution may be considered satisfactory.

According to the work of Clark and Lubs (1915), *E. coli* increases the hydrogen ion concentration of sugar broths so rapidly that the limit of tolerance is soon reached and growth stopped, while *A.*

aerogenes tends to decompose the acid as it is formed and consequently grows for a much longer time. It is to be remembered in this connection that a twenty-four-hour nutrient-broth culture of *E. coli* or a younger lactose-broth culture will reduce methylene blue. These facts indicate that a twenty-four-hour lactose-broth culture of *E. coli* does not reduce methylene blue because growth has entirely stopped or because the rate of growth is extremely slow. The senior author has other evidence to be presented in a later paper which shows that the rate of growth is a major factor in determining the potentials developed by cultures exposed to air.

SUMMARY AND CONCLUSIONS

1. Werkman's $FeCl_3$ modification of the Voges-Proskauer test was applied to twenty-four-hour cultures in standard glucose broth. The results were identical with those obtained by following the procedure outlined in the "Standard Methods for Water Analysis." The advantages of the proposed modification are as follows:

   a. The time required for the test is reduced from four days to twenty-four hours.
   b. Ordinary glucose broth can be used in place of carefully prepared media required for the older test.
   c. The results are more easily read in the proposed test than in the older one.

2. A methylene blue-reduction test is described for distinguishing between *E. coli* and *A. aerogenes* which agrees with the Voges-Proskauer test in 44 out of 45 cases. By making use of the lactose fermentation tube from the "completed test" for the coli-aerogenes group, the time required to distinguish between the two organisms is reduced to one hour and no special medium is required.

3. Potentiometric measurements were found to parallel the results obtained by dye reduction. It is pointed out that the rate of growth is a major factor in the development of potentials by cultures exposed to air.

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AVERAGING BACTERIAL COUNTS

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When a frequency distribution diagram illustrating a normal scatter of bacterial counts is made using the actual values, the curve assumes a distinct left-handed or J-shaped form. The peak of the curve and the arithmetic mean fall at quite divergent points. Furthermore, the standard deviation is usually much greater than, sometimes as much as twice as great as, the mean. Consequently a wide range of negative values is involved, indicating that something is radically wrong with the mathematical procedure. Statistical values based on an abnormal J-shaped distribution curve are unreliable.

Because the arithemtic mean does not reveal the true average in which the mean, median and mode nearly coincide, the writer has applied the geometric mean which is the value corresponding to the mean of the logarithms of the bacterial counts. A comparison of the two methods of averaging is submitted herewith.

The accuracy of an average is exceedingly important where the premiums which are paid to dairymen are based on an average of two or more bacterial counts of the milk as delivered. Likewise scientists when preparing growth curves based on an average computed from a series of similar observations may find it a valuable aid. Obviously the rate of bacterial growth is not constant. There are at least five phases in the normal growth curve in which the dairy bacteriologist is interested. These phases are: (1) the initial stationary or lag, (2) the positive accelerative, (3) the logarithmic or geometric, (4) the negative accelerative and (5) a stationary phase, prior to the decline when the death rate exceeds the birth rate. Notwithstanding the observation that geometric means among a series of samples in the logarithmic growth phase should approach a straight line relationship when plotted against time, the mere fact *per se* that bacteria increase geometrically, i.e., 1, 2, 4, 8, 16 and 32, instead of linearly, i.e., 1, 2, 3, 4, 5 and 6 justifies the application of the geometric mean.

The range of the standard agar plate counts for most milk is from 1,000 to 100,000,000 and undoubtedly 90 per cent of these counts are less than 100,000. To be sure, this extensive range overlaps the upper portions of the positive and the lower portions of the negative accelerative growth phases. However, the geometric mean so satisfactorily meets the requirement of a more accurate average as demonstrated in the charts and tables that its value for comparing different milk supplies or the same milk supply from year to year is justified. If it more nearly approaches the true average among a large number of counts, there is no reason to doubt this same tendency among two or three counts although its graphic demonstration is more difficult.

The averaging of logarithms is regarded as an improper mathematical procedure. This strict rule applies in most cases but was formulated long before biological science was known. Certainly, it originated before attention was called to the logarithmic growth phase among bacteria. If bacterial reproduction were a linear process, the arithmetic mean would be adequate. Because our common averaging system applies to linear dimensions, such as height, weight, length, capacity, etc., it is all the more difficult for the layman to appreciate the geometric increases among bacteria in their entirety. A rule 24 inches long is conceded to be twice the length of one 12 inches long. To regard a milk with a 20,000 count as twice as poor as one with a count of 10,000 is incorrect. Yet, this is exactly what we are doing when we regard 15,000 as the mean among the two values 10,000 and 20,000. Apparently workers in general have been slow to recognize when averaging bacterial counts that a bacterium reproduces by fission and makes two cells, each of which in turn splits giving four cells, etc. Partial recognition occurs when "Standard Methods" recommends reporting only the two significant left-hand digits in the bacterial count. Probably this recognition is wholly unintentional as the "Standard Methods" Committee has merely intended to simplify the report.

The problem then is how to transpose a geometrically increasing series to a linearly increasing series so that it can be averaged correctly. There are some who will regard this application as highly theoretical simply because they will not analyze mathematically the difference between an average among a series of values resulting from an arithmetic (or linear) progression and that resulting from a geometric (or logarithmic) progression. Table 1 illustrates some of these differences.

TABLE 1

A comparison of linear and logarithmic increases

UNIT OF TIME	LINEAR OR ARITHMETIC INCREASE	LOGARITHMIC OR GEOMETRIC INCREASE	LOGARITHMS CORRESPONDING TO THE GEOMETRIC INCREASE
0	10,000	10,000	4.00000
1	20,000	20,000	4.30103
2	30,000	40,000	4.60206
3	40,000	80,000	4.90309
4	50,000	160,000	5.20412
5	60,000	320,000	5.50515
6	70,000	640,000	5.80618
7	80,000	1,280,000	6.10721
8	90,000	2,560,000	6.40824
9	100,000	5,120,000	6.70927
10	110,000	10,240,000	7.01030
11	120,000	20,480,000	7.31133
12	130,000	40,960,000	7.61236
13	140,000	81,920,000	7.91339

If one can visualize the processes of bacterial increase during the logarithmic phase with respect to the unit of time (generation time) changes in table 1, one can readily see that during the first period the geometric increase was only 10,000. During the second period the actual increase was 20,000, during the third it was 40,000, during the fourth it was 80,000, etc. . . . . and during the thirteenth it was 40,960,000. Contrasted with this is the constant linear increase of 10,000 for each unit of time. With these seemingly enormous succeeding increases in the geometric series, is it any wonder that the bacterial counts are not more inaccurate than they are when it is realized that the increase from 40,960,000 to 81,920,000 theoretically requires just as long as the initial increase from 10,000 to 20,000? Robertson and Frayer (1930) have shown that these 13 units of time based on observations on 17 samples of milk held continually at 60°F., correspond to a 52 hour keeping quality interval, two hours after which all samples were considered as unfit as a sweet milk beverage.

If now, the bacteriologist will look at the last column in table 1, he will see listed the logarithms corresponding to values of the geometric increase. Since logarithms are powers of numbers calculated to the base 10, they are essentially linear values of measures representing a geometrically increasing series. The constant increase of log. 0.30103 represents a generation time and is equivalent to each 100 per cent increase in actual numbers of bacteria over the preceding count. Both the initial 10,000 increase during the first time unit and the 40,960,000 during the 13th time unit are represented by a logarithmic increase of log. 0.30103. The logarithms themselves, therefore, increase linearly while the values which they represent increase geometrically.

Table 2 illustrates a satisfactory system for the transposition of bacterial counts to logarithms and *vice versa.* Because of the character of the logarithmic spreads, it is necessary to establish two separate tables for these two transpositions. A certain degree of accuracy has been sacrificed in order to limit the logarithm to the second or third place following the decimal and the bacterial count to the first three significant left-hand digits. Therefore, the bacterial counts in the first part of the transposition table are the lowest values which can be read as equivalent to the logarithms. For instance 989 is the lowest value which can be read as equivalent to log. 2.995 and 1,010 as equivalent to log. 3.005, etc. When reading these logarithms in the second decimal place only, they become log. 3.00 and 3.01 respectively, etc. Hence all counts between 989 and 1,000 correspond to log. 3.00, all between 1,010 and 1,030 correspond to log. 3.01, . . . all between 9,440 and 9,590 correspond to log. 3.98 and all between 9,600 and 9,890 correspond to log. 3.99. Higher or lower bac-

TABLE 2

The transposition of counts to logarithms and vice versa

BACTERIAL COUNT AND CORRESPONDING LOGARITHM				LOGARITHM AND CORRESPONDING BACTERIAL COUNT			
989	3.00	3,130	3.50	3.00	1,000	3.50	3,160
1,010	3.01	3,200	3.51	3.01	1,020	3.51	3,240
1,040	3.02	3,270	3.52	3.02	1,050	3.52	3,310
1,060	3.03	3,350	3.53	3.03	1,070	3.53	3,390
1,080	3.04	3,430	3.54	3.04	1,100	3.54	3,470
1,110	3.05	3,510	3.55	3.05	1,120	3.55	3,550
1,140	3.06	3,590	3.56	3.06	1,150	3.56	3,630
1,160	3.07	3,670	3.57	3.07	1,180	3.57	3,720
1,190	3.08	3,760	3.58	3.08	1,200	3.58	3,800
1,220	3.09	3,850	3.59	3.09	1,230	3.59	3,890
1,240	3.10	3,940	3.60	3.10	1,260	3.60	3,980
1,270	3.11	4,030	3.61	3.11	1,290	3.61	4,070
1,300	3.12	4,120	3.62	3.12	1,320	3.62	4,170
1,330	3.13	4,220	3.63	3.13	1,350	3.63	4,270
1,360	3.14	4,320	3.64	3.14	1,380	3.64	4,370
1,400	3.15	4,420	3.65	3.15	1,410	3.65	4,470
1,430	3.16	4,520	3.66	3.16	1,450	3.66	4,570
1,460	3.17	4,620	3.67	3.17	1,480	3.67	4,680
1,500	3.18	4,730	3.68	3.18	1,510	3.68	4,790
1,530	3.19	4,840	3.69	3.19	1,550	3.69	4,900
1,570	3.20	4,950	3.70	3.20	1,580	3.70	5,010
1,600	3.21	5,070	3.71	3.21	1,620	3.71	5,130
1,640	3.22	5,190	3.72	3.22	1,660	3.72	5,250
1,680	3.23	5,310	3.73	3.23	1,700	3.73	5,370
1,720	3.24	5,430	3.74	3.24	1,740	3.74	5,500
1,760	3.25	5,560	3.75	3.25	1,780	3.75	5,620
1,800	3.26	5,690	3.76	3.26	1,820	3.76	5,750
1,840	3.27	5,820	3.77	3.27	1,860	3.77	5,890
1,880	3.28	5,960	3.78	3.28	1,910	3.78	6,030
1,930	3.29	6,100	3.79	3.29	1,950	3.79	6,170
1,970	3.30	6,240	3.80	3.30	2,000	3.80	6,310
2,020	3.31	6,380	3.81	3.31	2,040	3.81	6,460
2,070	3.32	6,530	3.82	3.32	2,090	3.82	6,610
2,110	3.33	6,680	3.83	3.33	2,140	3.83	6,760
2,160	3.34	6,840	3.84	3.34	2,190	3.84	6,920
2,210	3.35	7,000	3.85	3.35	2,240	3.85	7,080
2,260	3.36	7,160	3.86	3.36	2,290	3.86	7,240
2,320	3.37	7,330	3.87	3.37	2,340	3.87	7,410
2,370	3.38	7,500	3.88	3.38	2,400	3.88	7,590
2,430	3.39	7,670	3.89	3.39	2,450	3.89	7,760
2,480	3.40	7,850	3.90	3.40	2,510	3.90	7,940
2,540	3.41	8,040	3.91	3.41	2,570	3.91	8,130
2,600	3.42	8,220	3.92	3.42	2,630	3.92	8,320
2,660	3.43	8,410	3.93	3.43	2,690	3.93	8,510
2,720	3.44	8,610	3.94	3.44	2,750	3.94	8,710
2,790	3.45	8,810	3.95	3.45	2,820	3.95	8,910
2,850	3.46	9,020	3.96	3.46	2,880	3.96	9,120
2,920	3.47	9,230	3.97	3.47	2,950	3.97	9,330
2,990	3.48	9,440	3.98	3.48	3,020	3.98	9,550
3,060	3.49	9,600	3.99	3.49	3,090	3.99	9,770
		9,890	4.00			4.00	10,000

terial counts than those listed in table 2 may be transposed to the proper logarithms simply by increasing or decreasing the characteristic of the logarithm. For instance, if the count is 202 the log. is 2.31; if 20,200 it is log. 4.31; if 202,000 it is log. 5.31; etc.

In the second half of table 2, the values nearest the mean for each logarithm are listed. Because of the wide spread of values corresponding to these logarithms when shortened to the second or third[1] decimal place, it is necessary to employ the mean value corresponding to each logarithm.

Table 3 illustrates the use of the geometric mean when two counts are averaged. Because bacteria increase geometrically, a proportionate instead of a linear relation exists between counts on all samples of milk.

Where the difference between the counts is not great, the two means approach each other as in example 1, but where the difference is great, the geometric mean gives a lower average count, due to the proportionate relations. The linear relations in example 2 indicate that the 20,000 count milk is 20 times as poor as the 1,000 count milk. Counts from duplicate plates should always be averaged arithmetically.

In order to compare the two averaging systems to a better advantage, the standard agar plate counts and their corresponding logarithms for the 174 counts in table 4 are averaged. These counts, listed in the order of sampling one dairyman's night's milk four times monthly, were obtained through the courtesy of a New York State milk plant for the period beginning February 1, 1926, and ending December 31, 1929.

The arithmetic mean for the bacterial counts is 20,500 and the mean corresponding to the average log. 4.01 is 10,200. The mean 20,500 is far from the value corresponding to the peak of the curve. It is evident in chart 1 that 27 observations fall at the peak of the curve which is somewhere between 4,000 and 6,000. However, the long tail containing the five counts 96,000, 133,000, 136,000, 320,400 and 346,500, is of sufficient weight to bring the average up to 20,500. The mean is more than four times as great as the mode.

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[1] When averaging two counts only, it is advisable to use the counts corresponding to the additional logs. 3.005, 3.015, 3.025, 3.035, etc., because the sum of the logarithms frequently terminates with an odd digit. Failure to use this causes many of the geometric mean values to exceed the arithmetic means in cases where the two counts are relatively near each other. Obviously, they will exceed them in some cases but thus far the writer in applying the method has found no case where the dairyman has been placed at a premium disadvantage.

Now, if the logarithms of these same counts are used, there are 23 counts falling at the peak of the curve which is somewhere between 10,000 and 12,600. The distribution may be represented by a normal bell-shaped curve and both the mode and the mean approach each other very closely. Such being the case, does not the geometric mean reveal more accurately than the arithmetic mean the true distribution of a normal series of bacterial counts for market milk?

TABLE 3

Application and comparison of averages secured by the arithmetic and geometric mean

EXAMPLE NUMBER	ACTUAL BACTERIAL COUNTS AND ARITHMETIC MEANS	CORRESPONDING LOGARITHM AND GEOMETRIC MEANS
1	10,000	log. 4.00
	11,000	log. 4.04
	2)21,000	2)8.04
	Mean 10,500	Mean log. 4.02 = 10,500
2	1,000	log. 3.00
	20,000	log. 4.30
	2)21,000	2)7.30
	Mean 10,500	Mean log. 3.65 = 4,470
3	1,360	log. 3.14
	55,300	log. 4.74
	2)56,660	2)7.88
	Mean 28,400	Mean log. 3.94 = 8,710
4	5,000	log. 3.70
	15,000	log. 4.18
	2)20,000	2)7.88
	Mean 10,000	Mean log. 3.94 = 8,710

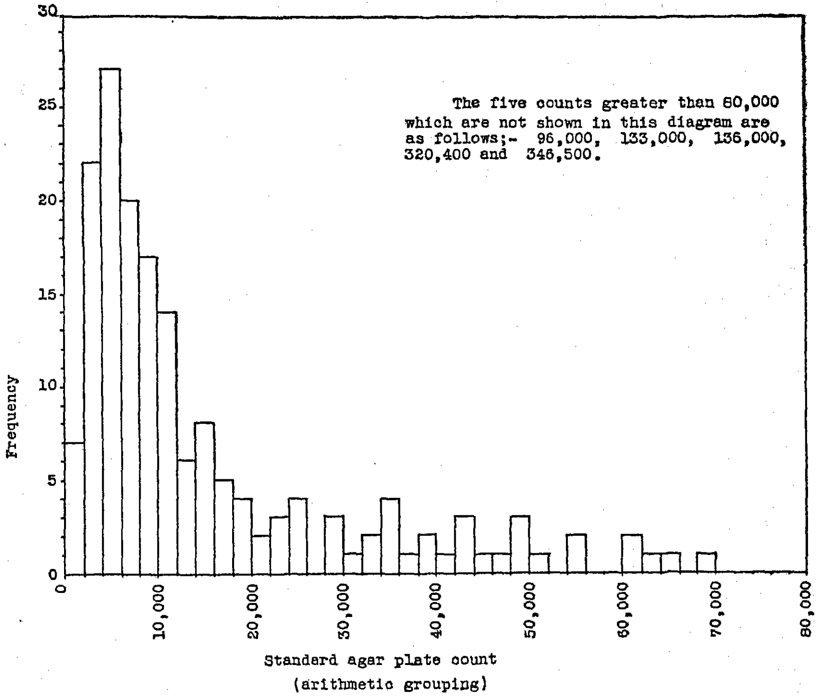
In order to emphasize the accuracy of this application, the

TABLE 4

Bacterial counts and corresponding logarithms for producer's night's milk

29,400	4.47	7,300	3.86	4,000	3.60	14,100	4.15
38,500	4.59	21,500	4.33	4,100	3.61	9,700	3.99
14,900	4.17	10,800	4.04	2,100	3.32	7,100	3.85
51,800	4.71	6,300	3.80	3,300	3.52	15,700	4.20
41,300	4.62	6,700	3.83	5,800	3.76	7,300	3.86
11,400	4.06	11,000	4.04	9,500	3.98	1,800	3.26
11,500	4.06	25,900	4.41	5,300	3.72	12,800	4.11
11,500	4.06	16,300	4.21	15,700	4.20	4,900	3.69
2,300	3.36	9,300	3.97	23,800	4.38	2,400	3.38
14,000	4.15	2,000	3.30	500	2.70	1,600	3.21
16,000	4.21	19,200	4.28	13,800	4.14	1,200	3.08
7,100	3.85	16,800	4.23	5,800	3.76	3,000	3.48
60,900	4.78	18,500	4.27	19,900	4.30	1,700	3.23
2,600	3.42	11,300	4.05	4,600	3.66	28,500	4.59
36,400	4.56	3,100	3.49	5,200	3.72	12,700	4.11
10,800	4.04	8,900	3.95	2,200	3.34	4,700	3.67
6,500	3.81	10,200	4.01	65,800	4.82	42,800	4.63
2,300	3.36	6,900	3.84	5,500	3.74	32,200	4.51
3,000	3.48	6,900	3.84	33,600	4.53	18,100	4.26
9,700	3.99	6,900	3.84	22,900	4.36	49,700	4.70
3,500	3.54	14,200	4.15	10,900	4.04	4,100	3.61
346,500	5.54	2,900	3.46	28,000	4.45	9,500	3.98
3,600	3.56	10,000	4.00	46,300	4.67	10,000	4.00
3,000	3.48	34,300	4.54	96,000	4.98	4,700	3.67
6,900	3.84	35,000	4.54	11,700	4.07	9,000	3.95
6,700	3.83	10,000	4.00	54,600	4.74	3,000	3.48
7,300	3.86	4,600	3.66	45,600	4.66	12,000	4.08
7,800	3.89	25,900	4.41	4,400	3.64	133,000	5.13
8,900	3.95	42,700	4.63	5,900	3.77	60,000	4.78
1,600	3.21	35,700	4.55	6,100	3.79	6,000	3.78
25,900	4.41	4,500	3.65	6,500	3.81	4,000	3.60
2,200	3.34	320,400	5.51	2,300	3.36	9,000	3.95
55,300	4.74	10,100	4.01	9,700	3.99	22,000	4.34
5,100	3.71	68,600	4.84	5,100	3.71	49,000	4.69
8,500	3.93	7,000	3.85	2,400	3.38	4,000	3.60
8,700	3.94	49,000	4.69	1,200	3.08	136,000	5.14
9,100	3.96	29,400	4.47	4,300	3.63	14,000	4.15
25,900	4.41	63,000	4.80	2,000	3.30	43,000	4.63
14,900	4.17	31,500	4.50	8,000	3.90	35,000	4.54
7,100	3.85	9,700	3.99	4,300	3.63	4,000	3.60
17,700	4.25	7,700	3.89	3,000	3.48	3,000	3.48
13,300	4.13	5,200	3.72	4,800	3.68	21,000	4.32
12,000	4.08	9,600	3.98	4,300	3.63	8,000	3.90
16,100	4.21	5,300	3.72				

writer invites attention to the facts as diagramatically shown by the respective standard deviations. The standard deviation among a series of values may be calculated most easily by the Ayer's method. This consists of extracting the square root of the difference between the average of the sum of the squares and the square of the average of the sum for any series of values. The empirical equation may be written:



The five counts greater than 80,000 which are not shown in this diagram are as follows;- 96,000, 133,000, 136,000, 320,400 and 346,500.

CHART 1. FREQUENCY DISTRIBUTION OF THE ACTUAL BACTERIAL COUNTS ON 174 SAMPLES OF NIGHT'S MILK AS DELIVERED BY A NEW YORK STATE DAIRYMAN AT A MILK PLANT WHERE PREMIUMS WERE PAID FOR LOW COUNT MILK

$$\sigma_x = \sqrt{\frac{\Sigma x^2}{n} - \left(\frac{\Sigma x}{n}\right)^2}$$

For the values in table 4, the standard deviation for the actual plate counts is ± 39,600 and for the geometric mean it is ± log.

0.48. In other words when using the arithmetic average 68.26 per cent of all the actual values should fall between −19,100 and +60,100. Could anything be more absurd than expecting from 10 to 15 per cent of all these bacterial counts to be less than zero?

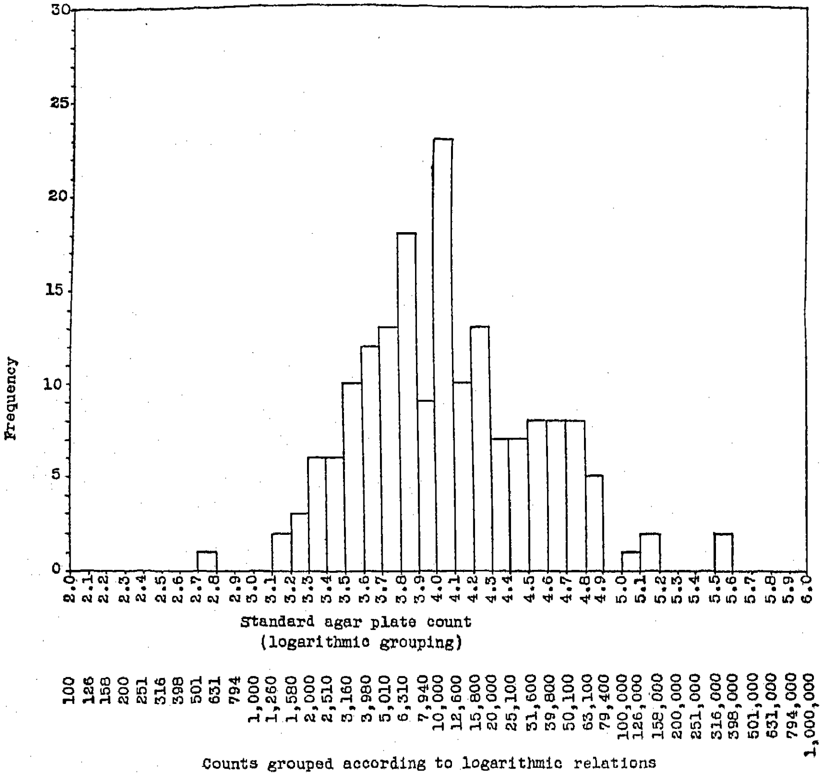


Chart 2. Frequency Distribution of the Logarithms of the Bacterial Counts on 174 Samples of Night's Milk as Delivered by a New York State Dairyman at a Milk Plant Where Premiums Where Paid for Low Count Milk.

If the standard deviation based on the logarithmic spread be used, the range is from log. 3.53 to log. 4.49. By transposing, these values become 3,390 and 30,900. Does this range not conform more closely with the known range and distribution in chart 2? Other statistical values merely confirm the ones herein described.

Other scientists have recognized the fallibility of the arithmetic mean and consequently have grouped bacterial counts instead of averaging them. Unfortunately, their grouping systems have not always taken into consideration the proportionate or geometric relations among counts. Breed and Stocking (1920) and Robertson (1921), have measured the relationships between counts by comparing ratios.

Frank (1927) was probably the first to apply the geometric mean to bacterial counts. Wholly unaware of the work of the United States Public Health Service, Robertson and Frayer (1930) demonstrated statistically the principles involved when the geometric mean is used. They have applied the geometric relations among counts in certain correlation studies also. As early as 1912, Dr. E. C. Levy (Commission (1912)) realizing the unreliability of the arithmetic mean, prepared a parabolic curve to register differences in bacterial counts. An arithmetic average among a series of class percentage values gave what he styled the "bacterial index." Kelley and Possam (1926) have recommended Levy's scheme to develop a more satisfactory way of averaging bacterial counts in milk and cream quality contests.

CONCLUSIONS

The geometric mean is a more nearly correct average than the arithmetic mean over a normal distribution of bacterial counts in market milk. In other words, the arithmetic mean when applied to the actual values in an exponential increase for determining the true average is unreliable.

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STUDY OF BACTERIAL FLUORESCENCE IN VARIOUS MEDIA

II. THE PRODUCTION OF FLUORESCENCE IN MEDIA MADE FROM PEPTONES

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INTRODUCTION AND HISTORICAL

In a previous communication the authors (1931) have shown that sulphates, phosphates, and magnesium must be present, in addition to an organic constituent, for the production of bacterial fluorescence in synthetic media by members of the *Pseudomonas fluorescens* group of bacteria. A number of peptones were used for making nutrient broth for the study of these organisms. The media made from some peptones produced fluorescence, whereas those made from other lots of peptones did not show pigment production.

Gessard (1892) studied the production of pigment in peptones using *B. pyocyaneus*. He concluded that a peptone, in order to produce fluorescence, must contain decomposed lecithine. Lepierre (1895) studied a pathogenic fluorescing bacillus in peptones made from various protein materials. He stated that fluorescence was due to meat extractives, such as xanthine and creatinine, plus soluble albuminoids.

The object of the research reported in this communication was to determine which peptones were suitable for use in media for the production of fluorescence; and also to endeavor to find what materials, necessary for pigment production, were lacking in the peptones which did not produce fluorescence.

TABLE 1

List of peptones used

NUMBER OF PEPTONES	MANUFACTURERS	KIND OF PEPTONE	pH VALUE
1	Arlington Chemical Company	Aminoids peptone (Beef)	5.70
2	Arlington Chemical Company	Aminoids peptone (Milk)	6.70
3	Armour and Company	Peptonum Siccum	5.50
4	Armour and Company	Peptonum Siccum	6.10
5	Armour and Company	Peptonum Siccum	6.05
6	Armour and Company	Peptonum Siccum	6.30
7	Baker, J. T.	Peptone, Bacto "Baker's"	6.95
8	Digestive Ferments Company	Bacto-peptone	7.00
9	Digestive Ferments Company	Bacto-peptone	6.45
10	Digestive Ferments Company	Bacto-peptone	6.90
11	Digestive Ferments Company	Bacto-peptone	6.90
12	Digestive Ferments Company	Bacto-peptone	6.52
13	Digestive Ferments Company	Bacto-peptone	6.91
14	Digestive Ferments Company	Proteose peptone	7.05
15	Digestive Ferments Company	Proteose peptone	6.92
16	Digestive Ferments Company	Proteose peptone	6.77
17	Digestive Ferments Company	Proteose peptone	6.90
18	Digestive Ferments Company	Proteose peptone	6.65
19	Digestive Ferments Company	Proteose peptone	7.05
20	Eimer and Amend	Peptone from albumin	6.21
21	Eimer and Amend	Peptone from beef	4.40
22	Fairchilds Bros. and Foster	Peptone	5.10
23	Fairchilds Bros. and Foster	Peptone	4.88
24	Fairchilds Bros. and Foster	Peptone	5.15
25	Merck and Company	Peptone Merck meat	4.50
26	Merck and Company	Peptone Merck meat	4.63
27	Parke, Davis and Company	Bacteriologic peptone	6.45
28	Parke, Davis and Company	Peptone	5.82
29	Parke, Davis and Company	Bacteriologic peptone	5.85
30	Parke, Davis and Company	Bacteriologic peptone	5.85
31	Special Chemical Company	Peptone Pfanstiehl	7.00
32	Squibb and Sons	Meat peptone	6.82
33	Squibb and Sons	Meat peptone	6.73
34	Squibb and Sons	Meat peptone	6.83
35	Stearns and Company	Peptonum Siccum	4.90
36	Stearns and Company	Peptonum Siccum	4.85
37	Stearns and Company	Peptonum Siccum	6.00
38	Witte, Rostock	Peptonum Siccum	6.95
39	Witte, Rostock	Peptonum Siccum	7.00
40	Witte, Rostock	Peptonum Siccum	7.18
41	Witte, Rostock	Peptonum Siccum	7.05
42	Witte, Rostock	Peptonum Siccum	6.92

METHODS AND MATERIALS

The methods used were similar to the ones previously used by the authors (1931). The peptone broth was made with 0.5 per cent peptone and 0.5 per cent potassium chloride. This was sterilized by the discontinuous method for thirty minutes each day for three successive days. The organisms used in this study were *Pseudomonas fluorescens* or closely allied species. A large number of peptones were used during the course of this research. These are listed in table 1, together with the pH of the unadjusted medium made from each peptone. Where there is more than one sample from a given manufacturer, each sample was of a different lot number.

TABLE 2

The effect of pH on pigment production in peptone broth

pH VALUES	1 DAY	2 DAYS	3 DAYS	4 DAYS
6.1	–	–	++	+++
6.5	–	–	++	+++
6.8	–	++	++++	++++
7.1	–	++	++++	++++
7.3	–	++	+++	++++
7.7	–	++	++	+++
8.1	–	+	++	+++

EXPERIMENTAL

In order to determine the effect of the pH on the production of fluorescence a series of media, having pH values ranging from 6.1 to 8.9, were prepared from one of the peptones which showed good pigment production. The results for one of the organisms used are shown in table 2. In this table, as well as those which follow, the relative amount of pigment produced is indicated by means of plus signs, four plus signs being the maximum. A negative mark indicates no fluorescence. In every case where there was lack of pigment production, the growth was good. Lack of fluorescence could not, therefore, be charged to absence of growth. The figures in parentheses indicate the time in hours required for the production of the maximum fluorescence.

TABLE 3

Fluorescence produced in media made from different peptones

NUMBER OF PEPTONES	ORGANISM A	ORGANISM B	ORGANISM C	ORGANISM D
1	++ (60)	+++ (24)	+++ (24)	+ (84)
2	–	–	–	–
3	–	–	–	–
4	–	–	–	–
5	–	–	–	–
6	–	–	–	–
7	–	–	–	–
8	–	–	–	–
9	–	–	–	–
10	++ (84)	+++ (48)	++++ (36)	+ (84)
11	–	+++ (36)	+++ (24)	–
12	++ (96)	+ (48)	++ (108)	+ (60)
13	+ (84)	++ (48)	–	+ (60)
14	+++ (24)	++++ (24)	++++ (36)	++++ (24)
15	++++ (24)	++++ (24)	+++ (36)	+++ (24)
16	+++ (24)	++++ (36)	+++ (36)	+++ (24)
17	+++ (24)	++++ (36)	+++ (24)	++++ (24)
18	++++ (24)	++++ (36)	+++ (24)	++++ (24)
19	++++ (24)	++++ (36)	+++ (24)	+++ (24)
20	+ (108)	+ (96)	–	–
21	+++ (24)	+++ (36)	+++ (24)	++++ (24)
22	+++ (24)	++++ (24)	+++ (24)	++++ (24)
23	+ (60)	–	–	+ (84)
24	+++ (96)	+++ (48)	+ (48)	++ (108)
25	–	–	–	–
26	–	–	–	–
27	–	–	–	–
28	++++ (48)	++++ (24)	+++ (36)	+++ (24)
29	+++ (60)	–	+ (48)	++ (84)
30	++ (72)	–	–	++ (84)
31	–	–	–	–
32	–	–	–	–
33	–	–	–	–
34	++ (96)	+ (48)	–	+ (108)
35	++ (48)	++++ (24)	+ (48)	++++ (24)
36	++ (60)	+++ (24)	+ (84)	+ (84)
37	+++ (24)	++ (36)	+++ (24)	+++ (24)
38	–	–	–	–
39	–	–	–	–
40	–	–	–	–
41	++ (48)	–	–	–
42	+ (144)	–	–	–

In table 2, we find that very good pigment formation was produced in media having a pH value of 6.1 to 8.1. The best production was, however, for the values between 6.8 and 7.3. In all of the media used, hereafter, the reaction was adjusted to a value between 6.9 and 7.1.

Each of the different peptones, as given in table 1, was used to prepare samples of media. Each medium was tested for ability to produce fluorescence with different organisms. The results for four of these organisms are recorded in table 3.

From the results given in table 3, we find seventeen peptones which produce no fluorescence with any of the four organisms. Slight fluorescence was produced by one or more of the organisms in ten peptones, whereas satisfactory fluorescence was produced in five peptones by two of the four organisms. Satisfactory fluorescence was produced in ten peptones by all four organisms. It will be noted that different strains will vary in their pigment production in the same medium.

A number of peptones whose media showed no pigment production were selected for further experimentation. Media were prepared from these peptones and various amounts of dipotassium phosphate added in order to determine whether this constituent was the one which was lacking for the production of pigment. Another set of media, containing varying amounts of magnesium sulphate, was prepared. A third set containing different amounts of these two constituents was also prepared. The results of these experiments for one of the six organisms used are listed in tables 4, 5, and 6.

In studying these three tables, one will notice that most of the peptones gave a good pigment production when phosphate was added. This was true with peptones numbers 7, 8, 9, 20, 23, 25, 27, 32, 33, 38, 39, and 40. A few of the peptones lacked both phosphates and magnesium sulphate. These included numbers 2, 4, 26, 31, and 42. Peptones numbers 2 and 4, however, did not give a very satisfactory fluorescence even when both constituents were added. Four of the peptones which produced a fairly good pigment on the addition of phosphates were improved somewhat by the addition of a small amount of magnesium sulphate. These were numbers 7, 27, 38, and 40. A number of the peptones would produce a good fluorescence in three or four days, but the pigment would fade in a day or two thereafter.

Peptones numbers 3, 5, and 6 showed practically no fluorescence when both phosphates and magnesium sulphate were added. It was thought that possibly these peptones lacked sufficient organic constituents with the nitrogen in an available form. Therefore, varying amounts of asparagine were added to media made from these peptones. No improvement, however, was noted. Several different amounts of asparagine were also added to the media made from each of the other peptones studied. Additions of this constituent were also made to the media prepared from each peptone containing added phosphates, magnesium sulphate, and the combination of the two. No improvement was noted in any of the above media.

TABLE 4

Pigment production in peptones with added phosphate—organism A

NUMBER OF PEPTONES	K_2HPO_4				
	0.001 per cent	0.01 per cent	0.025 per cent	0.05 per cent	0.1 per cent
2	−	−	−	−	++ (72)
3	−	−	−	−	+ (48)
4	−	−	−	−	+ (48)
5	−	−	−	−	−
6	−	−	−	−	−
7	−	−	+++ (72)	++ (72)	+++ (60)
8	+ (108)	++ (36)	+++ (48)	+++ (36)	+++ (24)
9	−	++ (60)	+++ (36)	++++ (36)	++++ (24)
20	++ (96)	+++ (48)	+++ (48)	+++ (48)	+++ (48)
23	−	++ (48)	++++ (24)	+++ (48)	++++ (48)
25	−	+++ (36)	+++ (24)	++++ (24)	++++ (12)
26	−	−	−	++ (48)	++ (48)
27	−	−	++ (60)	+++ (36)	+++ (60)
31	−	−	−	−	+++ (48)
32	−	++ (60)	+++ (24)	++++ (24)	+++ (24)
33	−	++ (60)	+++ (48)	+++ (24)	+++ (36)
38	−	+++ (48)	++ (60)	++ (60)	++ (48)
39	−	++ (60)	+++ (48)	+++ (48)	+++ (60)
40	−	+ (60)	++ (60)	+++ (48)	+++ (48)
42	−	++ (72)	−	++ (60)	++ (48)

According to Lepierre (1895) fluorescence is caused, in part, by meat extractives. A number of these extractives, and other closely related compounds, were added to the media made from the different peptones which showed no pigment production. Media were prepared containing various concentrations (0.001, 0.01, and 0.1 per cent) of these compounds. These media were tested for pigment production alone, and with the addition of phosphates and magnesium separately, and in various combinations. Sixty-six different media from each peptone were tested with four different organisms, but no marked improvement in fluorescence could be detected over a corresponding medium minus the special compound. The meat extractives and other compounds used were: caffeine, creatine, creatinine, guanine, theobromine, urea, uric acid, and xanthine.

TABLE 5

Pigment production in peptones with added magnesium sulphate—organism A

NUMBER OF PEPTONES	$MgSO_4$				
	0.001 per cent	0.01 per cent	0.025 per cent	0.05 per cent	0.1 per cent
2	–	–	–	–	–
3	–	–	–	–	–
4	–	–	–	–	–
5	–	–	–	–	–
6	–	–	–	–	–
7	–	–	–	–	–
8	–	–	–	+(120)	+(96)
9	–	–	–	–	–
20	–	–	–	–	++(72)
23	–	–	–	–	++(48)
25	–	–	–	+(60)	++(48)
26	–	–	–	–	–
27	–	–	+(60)	++(72)	++(60)
31	–	–	–	–	–
32	–	–	–	++(108)	++(96)
33	–	–	–	–	–
38	–	–	–	–	–
39	–	–	–	+(108)	+(72)
40	–	–	–	–	++(108)
42	–	–	–	++(96)	++(96)

The effect of heating, under different amounts of pressure, was next tried. Lepierre (1895) stated that heating under pressure destroyed the power of the medium to produce fluorescence. Media made from a number of peptones which produced no pigment alone were first tried. A small amount of phosphate and a large amount of magnesium sulphate were added to part of the media. In the other part the amounts of these constituents were reversed. These media were divided into different batches and heated under different pressures for varying lengths of time. The results are given in table 7. It was thought that the phosphate and magnesium might be rendered ineffective when magnesium ammonium phosphate was precipitated as the media were heated under pressure. However, the fluorescence in the media heated under pressure was nearly as good as in the media sterilized with live steam, except that the fluorescence was somewhat

TABLE 6

Pigment production in peptones with added phosphate and magnesium sulphate—organism A

NUMBER OF PEPTONES	0.001 PER CENT $MgSO_4$ 0.01 PER CENT K_2HPO_4	0.01 PER CENT $MgSO_4$ 0.01 PER CENT K_2HPO_4	0.05 PER CENT $MgSO_4$ 0.05 PER CENT K_2HPO_4	0.1 PER CENT $MgSO_4$ 0.1 PER CENT K_2HPO_4
2	–	–	++ (48)	+ (48)
3	–	+ (60)	–	+ (48)
4	–	+ (120)	++ (48)	++ (48)
5	–	–	–	–
6	–	–	–	–
7	–	+++ (48)	+++ (72)	+++ (48)
8	+++ (24)	+++ (24)	++++ (48)	++++ (48)
9	++ (60)	++++ (36)	+++ (48)	++++ (48)
20	+++ (72)	+++ (24)	+++ (48)	+++ (24)
23	+++ (48)	+++ (24)	++++ (48)	++++ (48)
25	+++ (24)	++++ (12)	++++ (24)	+++ (24)
26	–	+++ (48)	+++ (24)	+++ (48)
27	++ (60)	++++ (48)	++++ (48)	++++ (48)
31	–	++ (60)	++++ (48)	++++ (48)
32	+++ (36)	+++ (48)	++++ (48)	++++ (48)
33	++ (69)	+++ (48)	++++ (48)	++++ (48)
38	+++ (48)	++++ (48)	+++ (48)	++++ (48)
39	+++ (36)	+++ (48)	+++ (48)	+++ (48)
40	+++ (24)	++++ (24)	+++ (48)	++++ (48)
42	–	++ (48)	++++ (48)	+++ (48)

diminished in the media heated for thirty minutes under 25 pounds pressure. The fluorescence was exceedingly good in the media containing 0.1 per cent magnesium sulphate and 0.01 per cent phosphate when heated for twenty minutes under 20 pounds pressure.

TABLE 7

Effect on pigment production of heating under pressure of media made from peptones, magnesium sulphate and phosphate—organism A

NUMBER OF PEPTONE	0.1 PER CENT K_2HPO_4 + 0.01 PER CENT $MgSO_4$ ADDED			0.1 PER CENT $MgSO_4$ + 0.01 PER CENT K_2HPO_4 ADDED		
	Time and pressure used					
	15 pounds, 15 minutes	20 pounds, 20 minutes	25 pounds, 30 minutes	15 pounds, 15 minutes	20 pounds, 20 minutes	25 pounds, 30 minutes
3	+ (48)	+ (48)	–	+ (48)	+ (60)	–
9	++++ (48)	++++ (48)	+++ (48)	++++ (48)	++++ (36)	++ (48)
23	++++ (48)	++++ (48)	++++ (48)	++++ (36)	++++ (36)	++++ (48)
27	+++ (48)	+++ (48)	++++ (48)	++++ (36)	++++ (36)	++ (48)
33	++++ (48)	++++ (48)	++++ (48)	++++ (36)	++++ (48)	+++ (48)

TABLE 8

Effect on pigment production of heating under pressure of media made from peptones —organism A

NUMBER OF PEPTONE	TIME AND PRESSURE USED				
	Original*	15 pounds, 15 minutes	20 pounds, 20 minutes	25 pounds, 30 minutes	15 pounds, 60 minutes
16	+++ (48)	+++ (48)	++ (48)	+ (60)	++ (48)
17	++++ (24)	+++ (24)	+++ (48)	+++ (48)	+++ (48)
18	++++ (48)	++++ (24)	++++ (48)	+++ (48)	++++ (48)
19	++++ (48)	+++ (36)	+++ (36)	++ (48)	+++ (48)
21	+++ (48)	++ (48)	+++ (48)	–	++ (48)
28	+++ (48)	+++ (48)	++ (48)	++ (48)	+++ (48)
35	+++ (48)	+++ (48)	+++ (48)	+ (72)	+++ (48)
37	+++ (48)	++ (48)	+ (48)	–	+ (60)

* Sterilized in live steam, thirty minutes for three consecutive days.

Media were also prepared from several peptones which gave good fluorescence without the addition of other substances. These were sterilized as above. The results for one of the organisms with these media are reported in table 8. In general the pigment production was equally good in all media, with the exception that there was somewhat of a diminution at 25 pounds pressure.

In all media used up to this point the peptone content was 0.5 per cent. It was thought, perhaps, that this medium did not furnish enough food material in some of the peptones for pigment production. Media were prepared with each peptone using 3 per cent peptone and 0.5 per cent potassium chloride. These were tested as before. It was found that all of the peptones which failed to produce fluorescence with 0.5 per cent peptone also failed in the 3 per cent concentration. Also, in a number of the other peptones the pigment production was not as good as when there was less peptone, although the growth was as good or even better. Different concentrations of phosphate and magnesium sulphate, and combinations of these two, were added to the different media. The restoration of fluorescence, however, was not as good as with the media containing the lesser amount of peptone. Also, the addition of meat extractive and other substances did not increase pigment production in the media with the increased amount of peptone. It is thought that the greater concentration of food material promotes growth at the sacrifice of pigment production.

CONCLUSIONS

1. Peptones vary greatly in their composition and in their suitability for pigment production.
2. Some peptones lack the necessary amount of the different constituents which are necessary for pigment production. One may lack phosphate, another magnesium, etc.
3. On account of the variable composition it is necessary to test each lot of peptone for pigment production and then run a series of experiments to determine the constituent or constituents lacking so that these may be supplied to the medium.
4. A too concentrated medium will not satisfactorily support fluorescence even if the necessary constituents are present. A 0.5 per cent peptone broth is more satisfactory than a 3 per cent broth.
5. Purines, meat bases, and asparagine do not stimulate, to any great degree, the power of pigment production in peptones.

6. Heating peptone broth for a reasonable period of time under pressure, does not destroy the power to produce fluorescence.

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THE GROWTH RATE OF INDIVIDUAL BACTERIAL CELLS

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It has been assumed by many bacteriologists that during the period of rapid growth, in a satisfactory culture medium, some bacteria will die in spite of good food and favorable environment. No doubt this assumption was derived from an analogy with populations of higher forms of life, of which a number of individuals are known to die before they reach the reproductive age even with good care.

Wilson (1922) approached this question experimentally, and found that there was a regular discrepancy between plate counts and direct microscopic counts which he explained by supposing that some bacteria would not grow on agar but could still be seen. Reichenbach (1911), in order to explain the logarithmic order of death, assumed that a certain proportion of the multiplying bacteria of each new generation ceased multiplying and became dormant, and that the resistance of these dormant forms increased with age; thus a logarithmic gradation of resistance might be established.

These assumptions have been tested by us in the following manner: Bacteria, and in later experiments yeasts, from young liquid cultures were spread on the surface of an agar plate; from this seeded plate, a square was cut out and used as a hanging block in a moist chamber, for direct observation of multiplication under the microscope. The technique observed was essentially that of Orskow (1922), but the same field was kept under the microscope during the entire observation, which usually covered four generations. The moist chamber was held in a stage incubator where the temperature was maintained at 30°C., for all experiments.

A field was located in which there were 7 or 8 isolated cells and every five minutes a graphic sketch was made of all cell divisions,

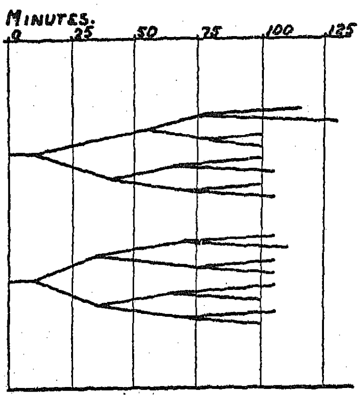

FIG. 1. *Bacterium aerogenes*

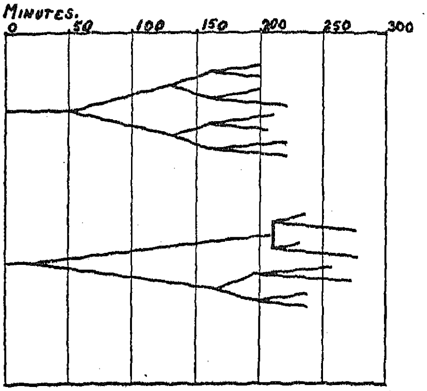

FIG. 2. *Bacillus cereus*

Chronological record of the growth of bacteria from one cell, where each branching indicates the time when a cell division occurred.

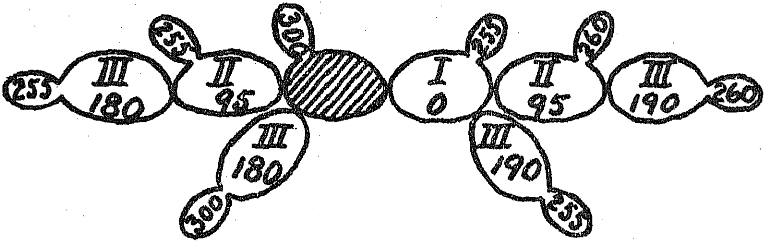

FIG. 3. YOUNG COLONY OF *Saccharomyces ellipsoideus*

The shaded cell is the mother cell. Roman numerals indicate successive generations, Arabic numerals mean "birth dates," i.e., the number of minutes when the cell appeared as a bud, taking the appearance of the first generation (cell I) as zero time.

similar to that shown in figure 3. In this manner the "family tree" was kept to the fourth generation, each original cell increasing from one to sixteen. Figures 1, 2 and 3 depict graphically the fission or budding times of the progeny of five typical cells.

Longer observation was impractical as it was not possible after this to tell from which mother cell the newly divided cells had come.

TABLE 1

Frequency of fission times of Bacterium aerogenes at 30°C., covering three generations

GENERATION	GENERATION TIME							INDIVIDUAL CELLS
	10–15 minutes	15–20 minutes	20–25 minutes	25–30 minutes	30–35 minutes	35–40 minutes	40–45 minutes	
II		3	8	4			1	16
III	2	3	13	12	2			32
IV	4	17	22	14	7			64

TABLE 2

Frequency of fission times of Bacterium aerogenes

	FISSION TIME														TOTAL
	5–10 minutes	10–15 minutes	15–20 minutes	20–25 minutes	25–30 minutes	30–35 minutes	35–40 minutes	40–45 minutes	45–50 minutes	50–55 minutes	55–60 minutes	60–65 minutes	65–70 minutes	70–75 minutes	
February 17					2	13	12	7	7	2	1				44
February 24			2	3	4	20	16	11	2	1		1			60
March 3		1	3	12	20	38	19	18	8	4	1	1		1	126
March 10			6	10	16	26	18	9	3	1				4	93
Total		1	11	25	42	97	65	45	20	8	2	2		5	323
March 2	1		3	19	22	21	10	5	2	1					84
March 6			4	12	30	19	15	1		2	1				84
Total	1		7	31	52	40	25	6	2	3	1				168
March 17		6	23	43	30	9		1							112
November 12			13	60	24	3									100
November 14			5	13	4	5	2	1							30
Total		6	41	116	58	17	2	2							242

Table 1 gives the detailed record of an experiment with *Bacterium aerogenes*. Eight cells were located in one field and these were found to divide between five and twenty minutes after observation had started. This table shows between which of the five-minute periods the second, third and fourth divisions took place. In this way many observations were made, summaries of which are given in tables 2, 3 and 4. In some cases, especially with *Bacterium aerogenes*, a lag was to be noted in the first recorded generation, and where this has occurred the data, for this generation, have not been recorded in tables 2, 3 and 4. It happened in 3 cases out of 110 that a cell did not divide in the new medium. An explanation for this might be found in the paper of K. A. Jensen (1928). But, if a cell divided once, all of its offspring continued to divide. There was not a single instance observed where a cell ceased multiplying, became dormant or died.

TABLE 3

Frequency of fission times of Bacillus cereus

	FISSION TIME																												
	0–5 minutes	5–10 minutes	10–15 minutes	15–20 minutes	20–25 minutes	25–30 minutes	30–35 minutes	35–40 minutes	40–45 minutes	45–50 minutes	50–55 minutes	55–60 minutes	60–65 minutes	65–70 minutes	70–75 minutes	75–80 minutes	80–85 minutes	85–90 minutes	90–95 minutes	95–100 minutes	100–105 minutes	105–110 minutes	110–115 minutes	115–120 minutes	120–125 minutes	125–130 minutes	130–135 minutes	135–140 minutes	TOTAL
March 24	3		1		6	2	8	7	9	18	16	5	6	3	5		1	2		1	1		1	1					96
March 30	2	2	2	4	5	8	8	11	5	6	3	1	6	2	2	2	1		2				1	1		1	1		76
April 1	4		1	1	6	5	4	5	4	10	6	6	9	3	5	3	5	1	2	1	2	1							84

TABLE 4

Frequency of budding times of Saccharomyces ellipsoideus

	FISSION TIME																											
	45–50 minutes	50–55 minutes	55–60 minutes	60–65 minutes	65–70 minutes	70–75 minutes	75–80 minutes	80–85 minutes	85–90 minutes	90–95 minutes	95–100 minutes	100–105 minutes	105–110 minutes	110–115 minutes	115–120 minutes	120–125 minutes	125–130 minutes	130–135 minutes	135–140 minutes	140–145 minutes	145–150 minutes	150–155 minutes	155–160 minutes	160–165 minutes	165–170 minutes	170–175 minutes	175–180 minutes	TOTAL
April 21	6			2		6	14	44	49	29	21	19	4	2	2	5	9	6	6									224
April 28												4	22	16	32	38	32	21	17	2	6		2	2				196

If the rate of death during "infancy" were the same for bacteria and yeasts as for the higher animals and plants, it should be possible to detect this by the method used providing a sufficient number of individual cells had been observed. Altogether 1,766 cells were kept under observation: *Bacterium aerogenes*, 977 individual cells; *Bacillus cereus*, 325 individual cells; *Saccharomyces ellipsoideus*, 464 individual cells.

It must be remembered, however, that this investigation covers only the first 4 generations after transfer to a new medium.

VARIATIONS IN THE RATE OF GROWTH

It is to be noted in tables 2 and 3 as well as on figures 1 and 2 that in the present investigation, considerable variation was observed in the rate of growth of individual cells under uniform conditions. This variation was shown not to be inheritable except for one yeast cell. In this case, six cells (table 4), all the progeny of one parent cell, were found to multiply in from forty-five to fifty minutes and 2 others from the same cell in sixty to sixty-five minutes, while the average was eighty-five to ninety minutes. This was the only example where fast growth appeared to be inherited. As a general rule, however, we do not have a mixture of fast-growing and slow-growing strains, but the two cells arising from the division of one cell may have greatly differing generation times. In table 5, the progeny of the fastest and the slowest growing cells of *Bacterium aerogenes* are shown to divide in a normal manner and are not greatly influenced by the dividing time of the parent cell. Since the progeny of the fastest cells multiplies not faster than the average, while that of the slowest cells is a little faster, it seems that the division of cell walls may be delayed after the cell contents have divided.

This is a common occurrence with spore-formers. A cell grows in length for a considerable time without dividing, and then when it finally divides, the next division comes very rapidly. This is to be seen in the second figure of figure 2, where one cell does not divide for one hundred eighty-five minutes, while the following division comes in twenty-five minutes. A more exaggerated example is to be found in the other half of the same figure, where the second and third divisions come at the same time interval.

In one case, with *Bacterium aerogenes*, the cell elongated without showing a division, and though the cell appeared as one, it continued to function as two cells. The next division occurred at the normal time. Each half of the cell divided as if it were an individual cell, breaking off a quarter of the entire length for the new cell. In the next generation the cell behaved again in the same way, while the offspring doubled normally. This cell might be compared to the "Siamese Twins."

TABLE 5

Fission times of the progeny of the fastest and slowest growing forms of Bacterium aerogenes

	FISSION TIME												
	5–10 minutes	10–15 minutes	15–20 minutes	20–25 minutes	25–30 minutes	30–35 minutes	35–40 minutes	40–45 minutes	45–50 minutes	50–55 minutes	55–60 minutes	60–65 minutes	65–70+ minutes
Progeny of the fastest growing cells													
February 17	—	—	—	—	—	—	1	—	1	—	—	—	—
February 23	—	—	—	—	—	2	—	4	1	—	1	—	—
February 24	—	—	—	—	—	9	3	2	—	—	—	—	—
March 2	—	—	—	12	2	—	—	1	1	—	—	—	—
March 3	—	—	—	1	3	7	2	2	—	1	—	—	—
March 6	—	—	2	—	3	8	4	—	—	1	—	—	—
March 10	—	—	—	—	4	9	—	1	1	—	—	—	3
March 17	—	3	5	5	2	1	—	—	—	—	—	—	—
Total	—	3	7	18	14	36	10	10	4	2	1	—	3
Progeny of the slowest growing cells													
February 17	—	—	—	—	—	4	1	1	2	2	—	—	—
February 23	—	—	—	—	—	—	2	—	7	—	—	—	1
February 24	—	—	1	1	—	—	—	—	—	—	—	—	—
March 2	1	—	1	1	—	—	1	1	—	1	—	—	—
March 3	—	1	2	3	—	—	—	—	—	—	—	—	—
March 6	—	—	1	2	1	—	—	—	—	—	—	—	—
March 10	—	—	2	3	1	—	1	—	—	—	—	—	—
March 17	—	1	2	1	—	—	—	—	—	—	—	—	—
Total	1	2	9	11	2	4	5	2	9	3			1

A chemical explanation of the cause of variation in growth rates of unicellular and multicellular organisms will be given elsewhere by one of the authors (Rahn, 1932).

SUMMARY

1. Microscopic observation of more than 1,700 individual cells of bacteria and yeasts showed that under favorable conditions of growth, all cells continue to multiply if they once have started to do so, and there was not a single case of "infant mortality" observed. The observations extended over the first 4 generations after transfer to agar.

2. There is considerable variation in the rate of fission (or budding) of the individual cells. This is not an inherent property of certain strains in the culture because the offspring of the fastest and slowest cells is not fast nor slow, but average.

The division of the cell contents (mitosis) is not always followed promptly by a division of the cell into two, and it may happen with spore-formers that one cell divides into 4 cells during the stage of fastest growth.

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BACTERIA IN ANTHRACITE COAL

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INTRODUCTION

During the past twenty years many papers have been written dealing with the presence of living bacteria in ancient rocks. Galle (1910–11), one of the earliest investigators in this field, states that bacteria are present in coal and are responsible for the production of mine gases such as methane and carbon dioxide. His conclusions, however, are subject to doubt due to the fact that he does not give the technique used in isolating the bacteria which he found. Schroeder (1914) substantiated Galle's findings of bacteria in coal but claimed that they did not produce gases. He stated that the bacteria got into the coal by infiltration from the surface. Whether this infiltration was due to cracks in the coal or to coal porosity is not made clear in his paper. The fact that he incubated his powdered coal for a week before growth appeared is, also, not consistent with our ideas of the reproduction of either spore formers or non-spore forming saprophytes.

Lieske and Hoffman (1929) in an experiment to determine at what depths in the earth's crust bacteria could live, found bacteria at a depth of 1089 meters. They found Gram-positive spore-forming bacteria and an occasional Gram-positive coccus in coal, but state that they were not the same as the 43 colonies caught on a petri dish exposed in the mine.

Lipman (1931) states that he found living bacteria in anthracite coal from Pennsylvania and that he believes that they have existed in the form of "invisible spores." The impression that he gives, that bacteria have existed in coal from carboniferous time to the present, does not seem logical or consistent with modern biological concepts.

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It is evident from fossil life that bacteria existed in very early time, probably long before the carboniferous period. Walcott (1914) explains the precipitation of pre-cambrian limestone as due to bacterial action. Ellis (1915 and 1919) in his work on iron deposits describes fossil forms of bacteria and fungi in various jurassic and cretaceous rocks.

Bastin and Greer (1930), Gahl and Anderson (1928), and Bastin (1930) explain the presence of hydrogen sulphide in oil wells as resulting from the reduction of sulphates by anaerobic bacteria which have presumably existed since early geological time.

Thiessen and Johnson (1929) have shown that the formation of peat is a microbiological process. Waksman (1930) in an analysis of a low moor peat profile, shows an decrease of aerobic microorganisms and an increase of anaerobic bacteria with increase in depth.

The present study was undertaken with the object of throwing more light on the subject of living bacteria in rocks of the past. It was thought that it might also show some relation of bacterial metabolism to the high and low volatile content of coal, and to the production of mine gases. It was further realized that this study might give an entirely new explanation of the progressive metamorphism of coal from peat to anthracite.

COLLECTION AND PREPARATION OF SAMPLES

The writers collected numerous samples of anthracite from the Primrose vein in the Otto colliery of the Philadelphia and Reading Coal and Iron Company at Pottsville, Pennsylvania. This location was selected because it was the same one from which Lipman's samples were secured. Samples were taken from the sixth level (east and west gangways) and the ninth level, approximately 300 and 400 feet underground. The Primrose vein in this colliery is in the form of a pitching syncline outcropping at the surface. The outcrop is badly breached, giving free access to surface waters.

Samples of solid coal, free from pyrite and bone, were taken from a position about 1 foot back of the exposed coal face. Several of these fresh samples were placed in large cans of formalin and transported to the laboratory; others were taken to the laboratory in an untreated condition to be used as controls. In later sampling, no germicide was used, for experimental work showed it to be unnecessary. In addition to coal samples, specimens of water and dirt were collected at each level. Samples of water and surface soil were also taken at the mine entrance.

In the laboratory, the lumps of coal were placed in an inoculation chamber where pieces ½ inch square were cut out of the center of each large sample by means of sterile instruments. These small pieces were ground to a fine powder in a mortar and taken up in 100 cc. of sterile distilled water in order to transfer directly into liquid media. All glassware, pestles, and mortars were sterilized in a hot air oven at 175°C. for two hours.

PROCEDURE

In the first series of tests the prepared samples were plated out on agar and into lactose broth. An examination for growth was made after forty-eight hours of incubation at a temperature of 37.5°C. Incubation was continued for twenty days with frequent examinations for growth. In all experiments the control was an uninoculated petri dish of a solid medium and a flask or test tube of a liquid medium. The results of these first tests are shown in table 1, in which no growth is recorded from any of the coal samples, but an abundant growth from the soil and water samples.

The failure to secure growth from the coal samples suggested the thought that bacteria, if present, might be autotrophic forms, obligate anaerobes, or other bacteria requiring special metabolites. For this reason, a series of media favorable to the growth of such selective organisms was utilized. These media were prepared from chemicals of known purity, adjusted colorimetrically to a hydrogen ion concentration of approximately 10^{-7} and sterilized at 15 pounds for twenty minutes. A liter of each medium was prepared in flasks, eight flasks being inoculated and two kept as controls. With solid media, four petri dishes were inoculated and a fifth kept as a control. The remainder of the previous samples of coal and some additional coal taken from the same locations were tested for growth.

TABLE 1

	TOTAL COUNT ON AGAR	GROWTH ON LACTOSE BROTH
9th level coal and formaldehyde	0	0
Ninth level coal	0	0
Ninth level mine water	2,925	Acid and gas
Ninth level mine dirt	195,000	Acid and gas
Sixth level west coal and formaldehyde	0	0
Sixth level west, coal	0	0
Sixth level mine water	19,900	Acid and gas
Sixth level dirt	3,500,000	Acid and gas
Surface water	3,950	Acid
Surface soil	1,300,000	Acid and gas

TABLE 2

1. Winogradsky's nitrogen-free medium
2. Peptone mannitol solution
3. Czapek's nitrate sucrose medium
4. Actinomyces medium (Waksman)
5. Potassium nitrite solution
6. Ammonium chloride solution
7. Lebediff's medium
8. Ruhland's medium
9. Glucose agar
10. Galle medium
11. Galle medium plus 0.1 per cent glucose
12. Galle medium plus 0.3 per cent toluene
13. Galle medium plus 0.3 per cent xylene
14. Galle medium plus 0.3 per cent wood charcoal
15. Galle medium plus 0.3 per cent blood charcoal
16. Galle medium plus 0.3 per cent vaseline
17. Galle medium plus 0.3 per cent lactic acid

These seventeen different media, shown in table 2, were examined for growth at various intervals over a period of fourteen days. Again negative results were obtained.

A more conclusive experiment was planned in which it was decided to inoculate 150 flasks of nutrient broth with a larger inoculum. A request was sent to the colliery for a large sample of coal to be taken from the same place from which we secured our first samples. When this coal arrived at the laboratory, it was noted that it contained a layer of bone and pyrite and was filled with minute fracture cracks. A piece chiselled out of the center of the lump was ground to a powder under the same sterile conditions previously observed. By means of a sterile spatula, 0.1 gram amounts were placed in 140 flasks, the remaining ten being controls. One half of the flasks were incubated at 25°C. and the remainder at 37.5°C. Contrary to our first experiments, growth occurred after twenty-four hours in all flasks with the exception of the controls. Two forms only could be isolated from these growths. The first and most abundant of these was a Gram-positive coccus; the second was a Gram-positive spore-forming rod similar to *Bacillus megatherium*, except in its action on lactose and nitrates.

TABLE 3

SAMPLES	TOTAL COUNT ON AGAR	GROWTH ON LACTOSE BROTH
Sixth level east, coal	426	Acid and gas
Sixth level east, mine water	3	Acid and gas
Sixth level east, mine dirt	700	Acid and gas
Sixth level west, coal	0	0
Sixth level west, mine water	375	Acid and gas
Sixth level west, mine dirt	1,125	Acid and gas

A visit to the mine, to learn the source of this bacteria-rich coal sample, disclosed that a mine boss had taken the sample from the sixth level but in a new opening. An examination of the coal in this area showed it to be fractured to a much greater extent than the coal formerly secured from the ninth and sixth levels. This highly fractured condition of the coal suggested that here might be the explanation of the presence of bacteria.

A new set of samples were collected from openings off the east and west gangways of this sixth level. Samples of solid and fractured coal were also taken from many other parts of the mine.

Samples of the dirt and mine water were taken from the sixth level. Surface water was also sampled from a creek a quarter of a mile from the mine. The results of tests for bacteria in these new sixth level samples are shown in table 3.

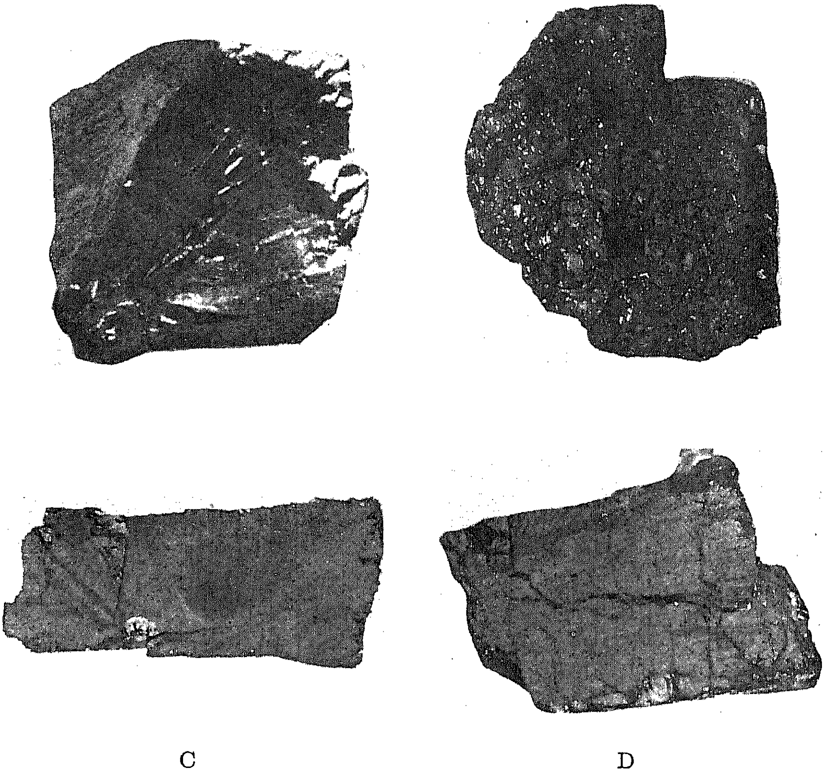

FIG. 1. A. A specimen of anthracite showing high conchoidal fracture but no cracks.

B. A specimen of anthracite showing many rectangular fracture cracks.

C. A specimen of anthracite cut in half, showing few fracture cracks.

D. A specimen of anthracite cut in half, showing many fracture cracks.

The absence of bacteria in the samples from the sixth level west is consistent with the fact that this coal was very compact while the coal from the sixth level east was filled with minute fracture cracks. Figure 1 shows photographs of these coals before and after cutting with a hacksaw.

A series of tests were made on fractured and unfractured coal from this and other mines to show conclusively the relation of cracks to the presence of bacteria. It was difficult to find pieces entirely free from fractures, but when they were found, nutrient broth inoculated with them was in most cases sterile. The exceptions can be explained by the possibility that some of the apparently fracture-free samples contained fine cracks which were invisible to the naked eye. In all cases where cracks were noticeable, bacteria were found.

IDENTIFICATION OF BACTERIA

Thirty-four forms of bacteria were isolated from the many tests of mine and surface waters, and mine and surface soils. The thought was that similar forms should be found in the coal if our assumption of penetration through fractures was correct. Ten of these forms were pigment formers belonging to the genera Pseudomonas, Sarcina, and Rhodococcus. Seven of the ten belonged to the genus Pseudomonas. The family Coccaceae was represented by six species of the genera Staphylococcus and Micrococcus. Six spore-forming rods, two actinomyces, and one sulfur oxidizing organism were found.

Bacteria identical in all respects with the two forms found in the coal were isolated from these mine and surface soils and water. The mine water taken from the sixth level where bacteria were first found proved to be almost a pure culture of the Gram-positive coccus found in the coal. As previously mentioned, the bacteria found in the coal were a Gram-positive spore-forming rod and a staphylococcus.

Attention is called to the fact that the iridescent bacteria belonging to the genus Pseudonomas were found in the mine and surface waters, but not in the coal. This form is mentioned particularly because it is one of the forms reported by Lipman as being found in anthracite.

These data cast very serious doubt upon the impression given by previous investigators that living bacteria have existed in Pennsylvania anthracite from carboniferous time to the present. The fact that in many instances the coal was found to be sterile and that bacteria were found in all cases where the coal was greatly fractured indicates that previous investigators did not give sufficient consideration to the physical constitution of the coal.

CONCLUSIONS

1. Pennsylvania anthracite from the Primrose vein contains no bacteria other than common living forms which have found ingress through fracture cracks communicating with surface water and air.

2. The two forms found in the coal, a Staphylococcus and a Gram-positive spore-forming rod, are very plentiful in the mine and surface waters and soils.

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FURTHER OBSERVATIONS ON THE RELATION OF pH VALUE TO TOXICITY OF PRESERVATIVES TO MICROÖRGANISMS

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In a previous paper (1929) it was shown that much more sodium benzoate was required to prevent growth of yeasts, molds and bacteria at pH values near neutrality (pH 5 to 8) than in the distinctly acid range of pH 2.5 to 4.5. The rate of multiplication of yeast and the rate of yeast fermentation, used as criteria of the toxicity of sodium benzoate to yeast, were also markedly affected by the pH value. In that paper were cited several investigators who had found that disinfecting power (killing effect) of several reagents is a function of the pH value. In the present paper is given he results of our recent experiments conducted to determine the effect of pH value on the preservative action of several weak acids (or their salts) and of two other preservatives, sodium chloride and formaldehyde.

EXPERIMENTAL

In these studies, the approximate concentrations of the preservatives required at three different pH values to prevent growth of *Penicillium glaucum*, *Saccharomyces ellipsoideus* and a mixed culture of *B. aceti* and lactic acid bacteria were determined. In addition, the relation of pH value to the retarding effect of these preservatives on yeast fermentation was studied.

Apple juice was used as the culture medium and was adjusted to pH values of 2.5, 3.5 and 7.0 by acidification with citric acid or addition of sodium hydroxide. For determining the concentrations of preservatives required to prevent growth at the three pH values, test tubes were used as containers for the medium. An inoculum of one loopful of an active culture per tube was used. Growth or absence of growth was determined by visual inspection, except in borderline and doubtful cases in which visual inspection was supplemented by microscopical examination. Tubes were stored at room temperature for three months or longer.

TABLE 1

Relation of pH value to the concentrations of several preservatives required to prevent growth

PRESERVATIVE	pH VALUE	CONCENTRATION TO PREVENT GROWTH			
		S. ellipsoideus	Penicillium mold	Mucor mold	Mixed bacteria
		grams per 100 cc.	*grams per 100 cc.*	*grams per 100 cc.*	*grams per 100 cc.*
Sodium salicylate	2.5	0.02	0.02	0.04	0.02
	3.5–3.8	0.06	0.15	0.10	0.08
	7.0	3.0+	3.0+	3.0+	3.0+
SO_2	2.5	0.02	0.02	0.03	0.01
	3.5	0.08	0.06	0.06	0.03
	7.0	Above 0.50	Above 0.50	Above 0.50	0.100
Acetic acid	3.5	0.8–1.0	0.8–1.0	0.8–1.0	No data
	7.0	Above 4.0	Above 4.0	Above 4.0	No data
NaCl	2.5	14	16	18	No data
	3.5	20	20	20	No data
	7.0	20	20	20	No data
Formaldehyde	2.5	0.015	0.015	0.015	0.015
	3.5	0.030±	0.030±	0.030±	0.030±
	7.0	0.030±	0.030±	0.030±	0.030±

Note: While, in the table the term "SO_2" is used in order to conform to usual practice, sodium sulfite was the reagent added. Acetic acid as such was added at pH 2.5 and 3.5 whereas, potassium acetate was added at pH 7.0. Its equivalent as "acetic acid" is reported in the table; i.e., the concentration of acetic acid corresponding to the total concentration of acetate radicle appears in the table.

Portions of 100 cc. of the juice of the three pH values were inoculated with an active culture of *S. ellipsoideus* and the rate of fermentation was followed by daily determinations of loss in weight caused by evolution of carbon dioxide.

The relation of pH value to the preservative action of the several reagents studied is shown in table 1.

The data show that the preservative action of the weak acids (or their salts) salicylic acid, sulfurous acid and acetic acid is markedly affected by the pH value of the medium. Thus, at pH 7.0 more than 150 times as much sodium salicylate was required to prevent growth as at pH 2.5. These preservatives resemble sodium benzoate in their action as reported in the paper cited earlier in this report. The preservative action of sodium chloride and formaldehyde was only slightly or very moderately affected by the pH value. Evidently, the undissociated weak acids and not their ions are the preservative agents. With salt, osmotic action is probably the factor involved and with formaldehyde, some other factor.

TABLE 2

Effect of pH value on the retarding action of sodium salicylate on the rate of fermentation by S. ellipsoideus

(Loss in weight, grams per 100 cc.)

TIME	pH 2.5		pH 3.5		pH 7.0		
	0 gram in 100 cc.	0.02 gram in 100 cc.	0 gram in 100 cc.	0.04 gram in 100 cc.	0 gram in 100 cc.	0.08 gram in 100 cc.	2.0 grams in 100 cc.
days							
1	0.13	0	0.38	0	0	0	0
2	1.40	0	1 70	0	0.75	0.82	1.20
3	2.58	0	2.96	0	2.05	2.14	2.98
4	3.58	0	4.06	0.735	3.12	3.29	4.11
6	5.40	0.43	5.80	2.20	5.21	5.40	5.64
10	7.01	0.56	7.30	3.66	6.85	6.71	7.04
13	7.92	0.85	7.34	4.11	7.22	7.02	7.56
16	8.25	1.34	7.95	4.84	7.86	7.53	8.26
21	8.78	1.69	8.36	5.65	8.19	7.82	8.57
24	9.06	2.08	8.42	6.45	8.50	8.11	8.88
32	9.59	2.97	9.40	7.85	9.25	8.83	9.60
34	9.69	3.15	9.60	8.14	9.40	8.98	9.77
36	9.98	3.81	10.08	8.90	9.85	9.41	10.19

The retarding effect on fermentation of concentrations of the various preservatives insufficient to prevent growth completely varied in a manner similar to that of the concentration required to prevent growth. In order to conserve space, the data for only one preservative, sodium salicylate will be presented. (See table 2.)

At pH 7 none of the concentrations of sodium salicylate used appreciably affected the rate of fermentation. At pH 2.5 and 3.5 beginning of fermentation was delayed by small concentrations of salicylate and the rate of fermentation was retarded.

The results with acetic acid and sulfurous acid were similar to those reported for the salicylate; while the retarding action of sodium chloride and formaldehyde was but little affected by the pH value. At very low concentrations it was observed that most of the preservatives exerted an appreciable stimulating effect on the initial rate of fermentation; this effect possibly being due to stimulation of multiplication of the cells.

SUMMARY

The concentrations of the weak acids, benzoic, salicylic, sulfurous and acetic acids required to prevent the growth of typical microörganisms from fruit juices were found to be much greater at or near neutrality than in the acid range, pH 2.5 to 3.5. Their retarding action on fermentation was similarly affected. The preservative action and retarding effect of sodium chloride and formaldehyde on fermentation were but little effected by pH value.

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BACTERIA PRODUCING TRIMETHYLENE GLYCOL[1]

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Trimethylene glycol was first observed as a product of fermentation in 1881 by Freund. It was shown, later, to be a product of the spontaneous fermentation of soap lyes and assumed considerable importance in this respect because its presence impaired the quality of the glycerol prepared from these lyes, giving rise to a product of low specific gravity known as "light stuff." Moreover, the fermentation resulted in a considerable loss of glycerol.

Steps taken to eliminate the spontaneous fermentation of soap lyes have so reduced the supply of trimethylene glycol on the market as to create a demand by manufacturers of certain pharmaceuticals. Cheaper production would undoubtedly lead to the development of new and more extensive uses.

Comparatively little study of the chemism of the fermentative production of trimethylene glycol has been made, and no systematic investigation of the organisms responsible has come to the attention of the authors. Such a study constitutes the purpose of the present paper.

HISTORICAL SURVEY

The occurrence of trimethylene glycol, as a product of the fermentation of glycerol, was observed by A. Freund (1881), while attempting to prepare *n*-butyl alcohol by the method of Fitz (1877). Freund observed that the syrupy residue remaining after distillation of the fermented mixture could not be brought again into fermentation, even after the butyl alcohol had been completely removed by distillation. This material proved to be neither glycerol nor an isomer but a new product of fermentation, distilling between 210° to 220°C. Repeated distillations gave a liquid boiling between 216° and 216.5°C. at 736 mm. pressure, which, together with its specific gravity of 1.0536 (18°/4°) (boiling point by Reboul 216° and specific gravity 1.053 at 19°), showed it to be probably trimethylene glycol. The dichloride, bromide and iodide of the material were prepared.

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Freund believed that the trimethylene glycol was formed by the abstraction of oxygen from the middle hydroxyl group, but did not determine the exact mechanism. The yield of trimethylene glycol was usually between 10 and 20 per cent, and sometimes as high as 27 per cent. Freund's organisms were probably spore formers. He made no bacteriological study but undoubtedly his organisms are not related to the non-sporulating forms to be described.

A. A. Noyes and W. H. Watkins (1895) investigated the presence of trimethylene glycol in glycerol and succeeded in obtaining a liquid from "light stuff" which boiled between 214° and 217°C., and had a specific gravity of 1.056 (20°/15°). They identified this material as trimethylene glycol, and suggested that its presence was due to the saponification of the fat, and subsequent spontaneous fermentation of the glycerol, thus causing an appreciable loss of the latter.

The method of analysis developed by Cocks and Salway (1922) proved to be of value in the determination of the quality of glycerol, under the assumption that only glycerol (g), trimethylene glycol (x), and water are present. They found the apparent glycerol content (acetin value), and the specific gravity of the material, setting up the following equations:

$$\text{Specific gravity of mixture} = 1 + 0.00263\,x + 0.00053\,g$$
$$\text{Apparent glycerol} = x + 0.81\,g$$

The method was not applicable to materials containing less than 1 per cent of trimethylene glycol. Cocks and Salway believed that the occurrence of trimethylene glycol as an impurity in the "dynamite" glycerol was a source of danger. They sought to improve their approximation of yields in their previous work, which was in error due to the contraction of volume on mixing of the three constituents, glycerol, trimethylene glycol, and water. They, therefore, used mixtures of known content, and constructed tables by which the quantity of trimethylene glycol can be determined from the specific gravity of the mixture. This procedure was worked out in detail, and its accuracy checked by using known mixtures, to within 0.2 per cent of the true percentage of trimethylene glycol. The boiling point of trimethylene glycol was found to be 210° to 211°C., and the specific gravity 1.0554.

C. A. Rojahn (1919) suggested that, if the water present in glycerol is known, the amount of trimethylene glycol may be calculated from the specific gravity of the mixture. He determined the water content by drying 2 grams on asbestos, under reduced pressure, over P_2O_5, with care not to prolong the drying longer than forty-eight hours, as the glycol was somewhat volatile. He then constructed graphs and tables giving the percentage of glycol for the varying specific gravity of the mixture and its water content.

In reviewing the work of Noyes and Watkins (1896), Archibald Rayner (1926), concluded that the trimethylene glycol must be formed subsequently to the liberation of the glycerol in the case of that obtained from rancid fats. He found that fermentation took place more readily in a slightly alkaline medium. From the progress and products of the fermentation, it was concluded that there were three types of organisms fermenting glycerol: (*a*) acid producers, (*b*) gas producers, (*c*) trimethylene glycol producers. The gas producers were thought to cause a loss as high as 60 per cent of the glycerol. Following his previous experience, Rayner thought it advisable, in order to prevent the formation of trimethylene glycol, to keep the lyes as strong as possible, and to work them up as quickly as practicable. Rayner found that crude glycerol contained, usually, less than 1 per cent trimethylene glycol, occasionally 2 or 3 per cent. He also found an isomer, α-propanediol present. Rayner gave the properties of trimethylene glycol as a pale yellow, odorless liquid, with density 1.0554 20°/20° according to Cocks and Salway's method and boiling at 210° to 211°C. The material was described as being less viscous than glycerol, and its solutions were almost identical in freezing point with equivalents of glycerol solutions. Contrary to previous belief, he found the nitration product of trimethylene glycol to be quite stable and that the trouble experienced with dynamite containing it was caused probably by another impurity. He likewise suggested that trimethylene glycol might be manufactured on a large scale, using a Van Ruymbeke still for the distillation of the fermented liquors.

Braak (1928) in his excellent monograph on the fermentation of glycerol, has discussed in some detail the chemism of glycerol fermentation by organisms of the coli-aerogenes group. He succeeded in isolating from canal water, an organism which he named *Bacterium Freundii* which produced considerable quantities of trimethylene glycol from glycerol. Braak placed his organism among the coli-aerogenes "intermediates."

METHODS

For purposes of the present study, the experimental work was limited to the organisms broadly known as the "coli-aerogenes" intermediate forms. Following the work of Braak which indicated the close relationship of *Bacterium Freundii* to species of Escherichia and Aerobacter, we used horse, sheep, mouse and cow manures and various soils as sources for the isolation of organisms producing trimethylene glycol. Glycerol media were employed for enrichment because the glycol has been found only among the products of the fermentation of glycerol. It has never been reported among the dissimilation products of the carbohydrates. A glycerol enrichment medium of the following composition was employed:

20 grams glycerol
1 gram di-potassium phosphate
3 grams peptone or ammonium chloride
1 liter water

The medium was adjusted to pH 7.0 and sterilized for fifteen minutes at 20 pounds pressure.

The enrichment culture was smeared on a medium of the same composition with 1.5 per cent of agar added. From the glycerol medium colonies were transferred to sodium citrate agar slants:

3 grams sodium citrate
3 grams ammonium chloride
1 gram di-potassium phosphate
1 liter of water
15 grams agar

The citrate medium was used to eliminate typical *Escherichia coli* forms after they had not been shown to produce trimethylene glycol. Braak (1928) had previously shown *Esch. coli* to be unable to produce the glycol and we have as yet to obtain an organism producing trimethylene glycol which fails to utilize the citrate radical. Experience led us to believe that the true acetoin-producing bacteria do not form trimethylene glycol and, later, typically V–P positive forms were discarded.

The production of trimethylene glycol was determined in a medium containing: glycerol 20 grams, dipotassium phosphate 1 gram, ammonium chloride 3 grams, and water 1000 cc. The culture was adjusted to neutrality daily by the addition of sodium hydroxide, or calcium carbonate was added to the medium in excess before sterilization.

The fermentations were carried out in 6-liter flasks equipped with two-hole stoppers. Into one hole was inserted a small sterile cotton-plugged separatory funnel extending to the bottom of the flask; through the other was placed a cotton-plugged delivery tube for the discharge of gases. The flask and medium were sterilized for thirty minutes at 20 pounds pressure. Hydrogen was bubbled through until the flask had cooled to incubating temperature, thus displacing oxygen and providing anaerobic conditions. The flasks were inoculated with 30 cc. of a forty-eight-hour culture and incubated at 30°C. For purposes of analysis, fermentation was considered complete after a period of ten to twelve days. Determinations were made on 4.5 liters of medium.

Cultures found to produce trimethylene glycol were subjected to a detailed cultural and systematic examination.

Uric acid medium was made according to Koser (1918). The utilization of the formate ion was determined in only one instance, and, then, by replacing sodium citrate in Koser's (1923) citrate medium with 0.5 per cent sodium formate. Indol was determined in tryptophane broth after two, five and seven days using the Gorë test. The reduction of nitrate to nitrite was determined by the dimethyl-alpha-naphthylamine test and also by the standard alpha-naphthylamine test. The former is more satisfactory, in that the color of the positive test does not fade. Determinations were made daily for seven days.

The Voges-Proskauer test for the production of acetoin was conducted according to standard methods as well as by that proposed by Werkman (1930) employing ferric chloride. Motility was determined by the soft agar method and by means of the hanging drop. Flagellation was observed by the aid of Muir's modified Pitfield stain.

Trimethylene glycol was determined by removing insoluble salts by filtration and concentrating the liquor to one-third its volume by distillation. This concentrate was further reduced to one-fourth of its volume by vacuum distillation and soluble calcium salts were precipitated by oxalic acid. The calcium oxalate was filtered off and the liquor evaporated to a syrup on the water bath. The syrup was dissolved in alcohol (96 per cent) and the salts removed by filtration. The filtrate was then distilled under a 29-inch vacuum. Everything coming over up to 100°C. was discarded. The fraction between 110 to 130°C. was a clear liquid which was refractionated under vacuum and the portion coming over between 110° and 120°C. was collected separately.

This fraction was further purified by distilling at a constant temperature of 210°C. at atmospheric pressure. This distillate was weighed and identified by the preparation of the dibenzoate.

The glycerol used in the fermentation was subjected to the same procedure. No trimethylene glycol was ever found. The dibenzoate prepared by the use of Eastman trimethylene glycol was compared with the fermentation product.

Appreciation is expressed for transplants of cultures received as follows:

V33B, M8B	Dr. S. A. Koser, Chicago University
Br	Dr. A. J. Kluyver, Delft, Holland
21R, 23R, 24R, 26R, 28R	Dr. C. C. Ruchhoft, Sanitary District of Chicago
17, 18	Dr. Roger Patrick of this laboratory

EXPERIMENTAL RESULTS

During the initial stages of the work, the scope was limited to a study of the "coli-aerogenes" group; it was subsequently restricted to the Gram-negative, non-sporulating rods fermenting lactose and glycerol and utilizing the citrate radical as a sole source of carbon. Only in the latter and more limited group were organisms found which produce trimethylene glycol. It was not found to be produced by typical *Escherichia coli* or *Aerobacter aerogenes*. All of the twelve cultures which were found to produce the glycol belong to a group generally known as the coli-aerogenes "intermediates" from the fact that they are methyl-red positive, Voges-Proskauer negative and fail to utilize uric acid as a sole source of nitrogen, but utilize citrate as a source of carbon. It becomes necessary to determine whether such "intermediate" forms are in fact to be allocated to Escherichia or to Aerobacter or should be grouped to form a new genus. These forms may well form a natural group deserving generic recognition.

It would be of distinct value in continuing the necessary studies to confirm the systematic position of these organisms, to have a simple, rapid qualitative test to detect the presence of trimethylene glycol in a culture medium. The close relationship of glycerol to trimethylene glycol makes the task of developing a test difficult. On the other hand, it may prove true that the production of trimethylene glycol will show a perfect correlation with cultural characteristics more easily determined.

CHARACTERIZATION OF CITROBACTER GEN. NOV.

All of the cultures examined for the production of trimethylene glycol belong to the coli-aerogenes group. Those found to produce the glycol are "intermediates" in this group, and show a homogeneity as a subgroup within the larger coli-aerogenes division. The trimethylene glycol-producing organisms will be characterized as a group as follows:

Morphological. Gram-negative, non-sporulating, short rods with rounded ends.

Physiological. The V-P test is negative with glycerol. In the case of two cultures (nos. 17 and 18) a faintly positive reaction is detectable with glucose after incubation at 30°C. for five days. The citrate ion is used as the sole source of carbon. The urate ion is not used as the sole source of nitrogen or serves poorly. In the case of culture 8 the urate medium shows some growth. Nitrates are reduced to nitrites. Milk is not peptonized.

Glycerol, mannitol, sorbitol, glucose, levulose, mannose, maltose, lactose, trehalose, xylose, rhamnose, and starch are dissimilated with the production of acid and gas. Erythritol, inulin and amygdalin are not dissimilated with the production of either acid or gas.

Biochemical. Trimethylene glycol, lactic acid, acetic acid, formic acid, CO_2, H_2, ethyl alcohol and succinic acid produced from glycerol; 2,3-butylene-glycol, acetoin, and acetone not produced from glycerol.

The methyl-red test does not prove satisfactory for the differentiation of the genus because of the intermediate nature of the organisms. Their behavior is generally to produce a positive test but occasionally the results are not definite.

The genus will be diagnosed with the purpose in mind of including the coli-aerogenes intermediates utilizing the citrate radical, methyl-red positive or weakly so and not producing acetoin from glycerol or glucose or in traces only from the latter. The diagnosis will not be limited, for the present, to organisms producing trimethylene glycol although production of the glycol occurs with all the cultures so far examined.

Diagnosis. Gram-negative, non-sporulating short rods with rounded ends. Methyl-red positive or intermediate. Acetoin not produced from glycerol and rarely from glucose and then only in traces. Citrate radical utilized. Nitrates reduced to nitrites.

Many carbohydrates, alcohols and glucosides attacked with the production of acid and gas. Ammonium salts utilized as a source of nitrogen. Type species: The type species is *Citrobacter Freundii* (Braak) *comb. nov.*

ALLOCATION OF CITROBACTER GEN. NOV.

It is necessary to allocate the citrate-positive, coli-aerogenes intermediates for purposes of systematic reference. The group as diagnosed is accorded generic ranking along with Escherichia and Aerobacter in the family Bacteriaceae. The generic name Citrobacter is proposed.

The group shows considerable homogeneity as a whole, whereas the individual organisms show dissimilative differences in their fermentative reactions which permit well-defined species differentiations. In the differential diagnosis of the genus, it proves impracticable to use the characters employed in the separation of Escherichia and Aerobacter. Citrobacter is intermediate to these two genera in certain characters which serve to distinguish them, i.e., methyl-red test, and uric acid utilization. Even the Voges-Proskauer test must be used under standard conditions.

The organisms show luxuriant growth on citrate media which serves as a characterizing cultural reaction of the group.

Three allocations of the group may be considered: (1) to the genus Escherichia, (2) to a new genus (3) to the genus Bacterium as a subgenus. This assumes recognition of the genus Bacterium. Allocation to Escherichia is probably unwise from the point of view of the systematist as well as the sanitary worker. The marked use of the citrate radical as a sole source of carbon is opposed to allocation to Escherichia.

Guided by the present tendency to recognize the splitting of the genus Bacterium into several genera, it is logical to accord generic ranking to this group of Escherichia-Aerobacter intermediate microörganisms. They are definitely intermediate in characters which are of decisive value in characterizing these genera. Furthermore, we know from unpublished results, supported by the work of Braak (1928) that not only do the metabolic products of the trimethylene glycol forms produced from glycerol differ from those of other organisms, but that marked quantitative differences also exist when the substrate is glucose.

If future study should show the advisability of recognizing the genus Bacterium, Citrobacter would become a subgeneric name for the group. Systematic study of the intermediate forms is being continued.

DESCRIPTIONS OF SPECIES

A key to the species of organisms producing trimethylene glycol has been prepared. Individual descriptions of the species are given.

Key to the species of Citrobacter

A. Gelatin liquefied.
  b. Acid and gas from sucrose (18a) (18). *Citrobacter decolorans.*
  bb. No acid and gas from sucrose (48) (48a). *Citrobacter album.*
AA. Gelatin not liquefied.
  b. Acid and gas from sucrose.
    c. Acid and gas from aesculin and salicin.
      d. Acid and gas from galactose (Br) (17). *Citrobacter Freundii.*
      dd. No acid and gas from galactose (8) (8a). *Citrobacter diversum.*
    cc. No acid and gas from aesculin and salicin (55) (51). *Citrobacter anindolicum.*
  bb. No acid and gas from sucrose.
    c. Acid and gas from dulcitol. No acid or gas from raffinose (28R) (23R) (24R) (M8BK). *Citrobacter intermedium n. sp.*
    cc. No acid and gas from dulcitol. Acid and gas from raffinose (V33B). *Citrobacter glycologenes n. sp.*

Citrobacter Freundii (Braak) comb. nov.

Source. Canal water. (Br); Soil (17). Cultures: Br. received from Dr. A. J. Kluyver. Isolated By Dr. H. R. Braak in Kluyver's laboratory. No. 17 isolated by Mr. Roger Patrick in this laboratory.

Morphology. Medium: Sodium citrate agar. Age: Twenty-four hours. Temperature: 30°C. Form: Short rods. Arrangement: Single, in pairs, or short chains. Ends: Rounded. Motility: Motile with peritrichous flagella.

Staining reactions. Gram-negative.

Cultural characteristics. Agar streak: Smooth, grey, shining, filiform and butyrous. Litmus milk: Acid in two days. Reduced and subsequently coagulated in twenty days. Gelatin stab: Growth apparent; no liquefaction.

Biochemical characters. Weak production of indol. Acetoin not produced from glucose. Utilizes ammonium chloride as a source of nitrogen. Methyl red positive. Produces H_2S in lead acetate agar. Gelatin not liquefied. Catalase produced. Citric acid utilized. Uric acid not assimilated. Reduces nitrates to nitrites.

Fermentation reactions. Acid and gas from aesculin, arabinose, glucose, galactose, glycerol, inositol, lactose, levulose, maltose, mannitol, mannose, raffinose, rhamnose, salicin, sorbitol, starch, sucrose, trehalose, and xylose. Neither acid nor gas from amygdalin, dextrin, dulcitol (?), erythritol, glycogen, inulin, or melezitose.

Braak was aware that his organism was an intermediate and gave it the name *Escherichia Freundii* because it behaved more like *Esch. coli* than *Aerobacter aerogenes.* Braak's organism becomes *Citrobacter Freundii* and type species of the genus.

Culture 17 was isolated from soil by Mr. Roger Patrick (1929) working in this laboratory. The description of no. 17 shows good agreement with Braak's description of his organism. Braak considered that the ability of his culture to attack dulcitol was doubtful, Patrick reported it positive. We have found the results with dulcitol indecisive and unreliable. Culture 17 will show a slight production of acetoin after at least five days' incubation in glucose medium at 37°C.

Citrobacter album (*Schive, comb. nov.*)

Sources. Feces.

Cultures. 48, 48a

Morphology. Medium: Sodium citrate agar. Age: Twenty-four hours. Temperature: 30°C. Form: Short rods. Arrangement: Single, pairs, or chains. Ends: Rounded.

Motility. Motile, peritrichous flagellation.

Staining reaction. Gram-negative.

Cultural characteristics. Agar streak: Growth moderate, filiform, glossy, raised. Plain broth: No surface growth, cloudy. Litmus milk: Acid and coagulated. Gelatin stab: Gelatin liquefied.

Biochemical characters. Indol production doubtful. Acetoin not produced from glycerol or glucose. Nitrates reduced to nitrites. Ammonium chloride utilized as source of nitrogen. Methyl red reaction positive. Hydrogen sulfide produced. Catalase produced. Uric acid not utilized. Citric acid utilized as a source of carbon.

Fermentation reactions. Acid and gas from arabinose, glucose, dextrin, dulcitol, galactose, glycerol, lactose, levulose, maltose, mannitol, mannose, raffinose, rhamnose, sorbitol, starch, trehalose, xylose. No acid or gas from aesculin, amygdalin, erythritol, inulin, melezitose, salicin, starch and sucrose.

Citrobacter glycologenes n. sp.

Source. Feces.

Culture. V33B.

Morphology. Medium: Sodium citrate agar. Age: Twenty-four hours. Temperature: 30°C. Form: Short rods. Arrangement: Single, pairs, or chains. Ends: Rounded.

Motility. Motile with peritrichous flagella.

Staining reactions. Gram-negative.

Cultural characteristics. Agar streak: Growth moderate, filiform, raised, glossy. No change in medium. Plain broth: No surface growth, slight ring, cloudy, no odor. Medium unchanged after twelve days at 30°C. Litmus milk: Acid in two days, no other change after twenty days. Gelatin stab: Growth evident along line of puncture but no liquefaction after thirty days at 20°C.

Biochemical characters. Indol not formed. Acetoin not produced from glucose. Nitrates reduced. Uses ammonium chloride as a source of nitrogen. Methyl red positive. No production of H_2S. Gelatin not liquefied. Catalase produced. Uric acid not assimilated. Citric acid utilized. Formic acid not utilized.

Fermentation reactions. Acid and gas from glucose, galactose, glycerol, lactose, levulose, maltose, mannitol, mannose, raffinose, rhamnose, sorbitol, starch, trehalose, and xylose. No acid or gas from aesculin, amygdalin, arabinose, dextrin, dulcitol, erythritol, inulin, melezitose, salicin, sucrose.

Citrobacter intermedium n. sp.

Source. Feces (M8B).

Cultures. M8B, 23R, 24R and 28R.

Morphology. Medium: Sodium citrate agar. Age: Twenty-four hours. Temperature: 30°C. Form: Short rods. Arrangement: Single, pairs, or chains. Ends: Rounded.

Motility. Motile with peritrichous flagella. Usually two flagella at one pole.

Staining reactions. Gram-negative.

Cultural characteristics. Agar streak: Growth moderate, filiform, raised, glossy. No change in medium.

Plain broth: no surface pellicle, slight ring, cloudy, no odor, medium unchanged in appearance after twelve days at 20°C. Litmus milk: Acid in two days, followed by reduction. No coagulation. Gelatin stab: Growth evident along line of puncture but no liquefaction after thirty days at 20°C. Biochemical characters: Indol not formed. Acetoin not produced from glucose. Nitrates reduced. Uses ammonium chloride as a source of nitrogen. Methyl red positive. No production of H_2S. Gelatin not liquefied. Catalase produced. Uric acid not assimilated. Citric acid utilized. Formic acid not utilized.

Fermentation reactions. Acid and gas from arabinose, glucose, dulcitol, galactose, glycerol, lactose, levulose, maltose, mannitol, mannose, rhamnose, sorbitol, startch, trehalose and xylose. No acid or gas from aesculin, amygdalin, dextrin, erythritol, inositol, inulin, melezitose, raffinose, salicin and sucrose.

Citrobacter decolorans (Burkey) comb. nov.

Source. Soil.

Culture. 18, 18a.

Morphology. Medium, glucose phosphate peptone agar. Age: 24 hours. Temperature: 30°C. Form: Short rods (0.9μ by 1.6μ). Arrangement: Single or in pairs. Ends: Rounded. Spores: Absent. Capsules: Absent in milk.

Motility. Non-motile.

Staining reactions. Gram-negative. No granular appearance with iodine.

Cultural characteristics. Agar slant, growth moderate, filiform spreading, glistening, smooth. Plain broth: No surface pellicle, turbidity. Litmus milk: Acid after twenty-four hours, reduction after four days. Coagulation on tenth day. No peptonization. Gelatin stab: Line of puncture filiform growth. Liquefaction. Potato slant: Yellow-white, lustrous growth.

Biochemical characteristics. Indol formed. Nitrates reduced to nitrites. Ammonium salts utilized. Methyl red reaction intermediate to negative. H_2S produced. Gelatin liquefied. Diastase production slight. Weak test for acetoin from glucose after five days.

Fermentation reactions. Acid and gas from adonitol, aesculin, arabinose, glucose, galactose, glycerol, inositol, lactose, levulose, maltose, mannitol, mannose, pectin, raffinose, rhamnose, salicin, sorbitol, starch, sucrose, trehalose, and sylose. No acid or gas from amygdalin, arabitol, dextrin, dulcitol, erythritol, inulin and melezitose.

Culture (18) was isolated by Mr. Roger Patrick (1929) during studies on the fermentation of xylan. It agrees with one minor exception, in characteristics with the organism which Burkey (1928) isolated and named *Aerobacter decolorans*. Burkey (1928) in his description of the species states that acid and not acid and gas is formed in rhamnose medium. In his description of the individual cultures of *Aerobacter decolorans* he shows nos. 3 and 4 as producing acid but no gas but cultures 24, 30 and 31 are shown as producing both acid and gas from rhamnose. It gives a weak Voges-Proskauer positive reaction after five days' incubation in glucose medium. Its name therefore should be *Citrobacter decolorans comb. nov.*

Citrobacter diversum (Burkey) comb. nov.

Source. Soil (8); feces (8a).

Cultures. 8, 8a.

Morphology. Medium: Glucose-phosphate-peptone broth. Age: Twenty-four hours. Temperature: 37°C. Form: Short rods. Arrangement: Single or pairs. Ends: Rounded. Capsules: Capsulated in twenty-four-hour culture of litmus milk.

Endospores. Absent.

Motility. Non-motile.

Staining reactions. Gram-negative.

Cultural characters. Colony: Size 5 to 8 mm. in diameter, round semi-opaque, finely granular. Medium not discolored. Agar streak: Growth abundant, filiform, shiny and butyrous. Plain broth: Cloudy, with slight ring. Litmus milk: Acid and gas. No reduction or coagulation.

Biochemical reactions. Indol produced. Acetoin not produced from glucose or glycerol. Nitrates reduced. Uses uric acid as source of nitrogen and citric acid as sole source of carbon. Methyl red reaction indefinite. H_2S produced. Gelatin not liquefied. Diastase produced.

Fermentation reactions. Acid and gas from adonitol, arabinose, aesculin, glucose, glycerol, glycogen, inositol, lactose, levulose, maltose, mannitol, mannose, melezitose, pectin, raffinose, rhamnose, salicin, sorbitol, starch, sucrose, trehalose and xylose. Acid and gas not produced from amygdalin, dextrin, dulcitol, erythritol, galactose and inulin.

No. 8 is the original strain isolated and described by Burkey (1928) and called by him *Aerobacter diversum*. It becomes *Citrobacter diversum comb. nov.*

Citrobacter anindolicum (*Lembke*) *comb. nov.*

Source. Soil.

Cultures. 51 and 55.

Morphology. Medium, sodium citrate agar. Age: 24 hours. Temperature: 30°C. Arrangement: Single, pairs or chains. Ends: Rounded.

Motility. Motile with peritrichous flagella.

Staining reaction. Gram-negative.

Cultural characteristics. Agar streak: Growth fair, filiform, convex, glossy. Plain broth: No surface pellicle, slight ring, turbid. Litmus milk: Acid in two days. Gelatin stab: Growth along line of puncture but no liquefaction after thirty days at 20°C.

Biochemical characteristics. Indol not formed. Acetoin not produced from glycerol or glucose. Nitrates reduced to nitrites. Uses ammonium salts as a sole source of nitrogen. Methyl red positive. H_2S produced. Gelatin not liquefied. Catalase produced. Uric acid not assimilated. Citric acid utilized as sole source of carbon.

Fermentation reactions. Acid and gas from arabinose, glucose, dulcitol, galactose, glycerol, lactose, levulose, maltose, mannitol, mannose, raffinose, rhamnose, sorbitol, starch, sucrose trehalose and xylose. No acid or gas from aesculin, amygdalin, dextrin, erythritol, inulin, melezitose and salicin.

Bergey (1930) described *Escherichia anindolica* as coagulating milk. Cultures 51 and 55 agree with Bergey's description in all other characters.

CONCLUSIONS

Fifteen cultures of bacteria have been found to produce trimethylene glycol from glycerol. All of the cultures belong to the citrate-positive, coli-aerogenes intermediates.

It is proposed to recognize the group as diagnosed as a new genus in the family Bacteriaceae. The name *Citrobacter* is proposed for the genus.

Seven species are recognized in the genus; *Citrobacter Freundii* (Braak), *comb. nov.* is the type species.

Should the genus Bacterium be retained Citrobacter becomes the name of a subgenus.

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A NEW AND SIMPLIFIED MICRURGICAL APPARATUS ESPECIALLY ADAPTED TO SINGLE CELL ISOLATION

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With few exceptions the objections to mechanical micrurgy and especially to single cell isolation concern the complication of the manipulators as well as their prohibitive cost. Attempts to simplify the apparatus have resulted in a loss of accuracy and flexibility in many cases. Attempts made to improve mechanical single cell technique have often resulted in complicating the apparatus. In this article, the writer presents an apparatus, new in design, simplified in structure, and made especially for single cell isolations, but adaptable to any micrurgical technique. Because of the absence of close machine work the apparatus may be made easily and cheaply in any machine shop. The value of the simplicity in construction is evident when after a year of constant use by the students in the laboratories of Notre Dame, the particular model described in this paper shows no signs of wear and has lost nothing in accuracy.

In working with micro-manipulators, especially in relation to single cell isolations, it has become evident to the writer that the following features are necessary to successful work: (1) a quick adjustment for centering the pipette point in the microscopic field; this adjustment should be accurate and should not complicate the apparatus; (2) fine adjustments which are not too delicate and too sensitive; (3) limited movements so that the sweep of the pipette point is only as wide as a low power field; (4) a quick, convenient, and accurate method of replacing pipettes which is incorporated in the manipulator and not as an accessory to it.

While these things are necessary to successful mechanical single cell isolation, they are equally valuable in any micrurgical procedure whatever its nature. Since micro-manipulators are designed especially to work under the lens of a microscope, they should be refined to that end.

SURVEY OF MICRO-MANIPULATORS

Many instruments have been designed for manipulating micro tools. The following list is offered both as a matter of historical interest and to show how the mechanical principles employed by the writer in his apparatus, differ from those used by previous designers.

Schmidt (1859) used a direct screw-thread principle. The needle point was moved in arcs and a straight line, and movements were in three planes. Manipulations were performed between the cover slip and the objective, and the manipulator was mounted directly on the stage of the microscope.

Chabry (1887) designed an apparatus working in one plane (from side-to-side) using a worm-thread-lever principle which produced movements in a straight line. The instrument was mounted on a glass microscope slide, and the object worked upon was held in a capillary glass tube.

Doty (1900) used a screw-thread action pushing against a lever and a spring. The instrument was moved in one plane (up-and-down), in the form of an arc, and was attached directly to the objective. The needle point was manipulated between the cover slip and the objective.

Barber (1904, 1907, 1909, 1911, 1914) used micro-pipettes which he manipulated in a hanging drop. The manipulating apparatus worked in three planes and was of the direct screw-thread, traveling-block type, and was fixed to the stage of the microscope by a clamp.

Hecker (1916) improved Barbers' model by using gib take-up screws and replaceable steel shims to take up the wear on the bearing surfaces and thus rid the instrument of lost motion. His manipulator was double.

Malone (1918) employed a microscope stand to obtain his one plane movement (up-and-down) depending on the mechanical stage of the observation microscope for movements in the other two planes.

Bishop and Tharldison (1921) perfected a unique instrument which made use of a screw-thread-lever principle similar to the better known Chambers' apparatus. The movements were in three planes and in arcs. Work was done beneath a cover slip in the Barber manner. The apparatus was mounted on the microscope stage.

Chambers (1922) produced an instrument similar to the apparatus of Bishop and Tharldison, but differing from it in design. The principle used is screw-thread against a lever and a spring. Movements of the needle point are in the form of arcs in three planes.

Peterfi (1923) designed an instrument which used direct screw-thread, and imparted motions to the needle points in straight lines and at angles to each other.

Taylor (1923–25) employed a direct screw-thread principle which imparts movements to the needle point in straight lines. This apparatus was mounted apart from the microscope stage on a special base. It was made quite massive to withstand rough usage.

Johnson (1923) describes an apparatus movable in one plane (up-and-down). The action is in the form of an arc and is pivoted from a ball and socket joint.

Fitz (1931) describes an apparatus using a cone, thrust against a movable point. Movements in three planes are imparted to each plane by screw-thread action.

DESCRIPTION OF THE WRITER'S APPARATUS

The principle on which the writer's apparatus is based, is that of a floating center made possible by a ball and socket. Movements in three planes and in arcs are produced by the individual mechanisms or "movement cylinders." These cylinders may be operated separately to produce movement in one plane, or they may be combined to produce movements in three planes.

Each movement cylinder consists of a brass tube in which a ball and socket, carrying the pipette holder is moved, in an up-and-down direction by means of a screw point pressing against the socket which is, in turn, pressed against a spring. The movement is continuous and there is no play or looseness during adjustments. The end of the cylinder has a series of threads chased on the outside on which a cap similarly threaded is screwed. This cap is made of brass and has a teat turned on it. A hole is drilled longitudinally through the teat and tapped 80 threads to the inch. Midway on the length of the cylinder a slot is milled through both walls. The cylinder is closed on the uptapped end with a brass plug which has a teat turned on both sides of it. The teat on one side is to act as a seat for a spring, and the one on the other side is threaded and serves to mount the cylinder to a base.

The movement screw, which imparts motion to the ball and socket has a knurled head on one end, is threaded to fit the tapped hole in the cap (80 threads to the inch), and has a small steel ball sunk into its pushing end. This ball minimizes friction and secures smooth movement.

The socket-bearing which travels up and down the length of the cylinder is made of an upper and lower portion. Both portions are made alike in the form of small cylinders and are accurately turned to fit the bore of the movement cylinder. One side of the bearing cylinder is hollowed out to act as a seat for the ball; the other side of the cylinder is turned to a teat. The lower socket-bearing presses against a spring, which in turn presses against the plug in the bottom of the cylinder. The upper socket-bearing comes into contact with the steel ball at the end of the adjustment screw.

The steel ball on which the movements of the needle tip depends, is seated between the two parts of the socket-bearing. A hole is drilled through this ball and a piece of brass tubing is pushed through the hole. This brass tubing acts as a guide-sleeve for the pipette holder. The steel ball carrying the pipette holder fits between the two socket-bearings which press tightly against its surface by reason of the spring on one end and the movement screw on the other. It is obvious that movements may be obtained in any direction from this mechanism, but that movement is controlled in only one direction, up-and-down. Controlled movements in other planes must make use of combinations of these individual movement cylinders.

The apparatus presented in this model makes use of two of these movement cylinders to produce movement at the needle point in two planes. The third plane, in which the needle tip is moved from side-to-side on the microscope stage, depends on a simplified direct screw-thread movement.

One of the movement cylinders is mounted upright and the other horizontal to it so that the holes in the two balls are on the same level. The screw in the upright cylinder produces an up-and-down movement in relation to the stage of the microscope, using the ball in the horizontal cylinder as a pivot point. When the screw in the horizontal cylinder is turned, the movement is in a side-to-side plane, and uses the ball in the upright cylinder as a pivot point. This combination is essentially a floating center.

The third plane imparting motion in a back-and-forth direction is in a straight line and of a direct screw-thread, traveling-block principle. The screw has 80 threads to the inch and is anchored through the end of a plate and passes through the center of a slot milled in the plate. The traveling-block consists of two bearing plates which press against the upper and lower surfaces of the screw-bearing plate. Between these bearing plates a smaller block of the same width and thickness as the slot is fixed. A hole is drilled and tapped through this block to fit the screw. Any movement of this screw tends to impart a back-and-forth movement to the traveling-block.

The two movement cylinders are mounted on a metal block in the positions previously indicated. The block pivots on a plate by means of a steel axis fixed in back of the perpendicular movement cylinder. The plate on which the movement cylinders are mounted, pivots on the upper bearing-plate of the back-and-forth movement.

STAND FOR THE MICRO-MANIPULATOR

The stand represented in figure 2 consists of a pair of clamps which fasten to the stage of the microscope and which may be raised or lowered by two screws. Two adjustable, telescoping pillars are mounted on the back end of the manipulator to steady the apparatus against the table.

The stand used is represented in figure 1 and is adjustable for height as well as position in relation to the stage of the microscope. This stand consists of a ball and socket, which may be clamped into position once the instrument is set. The stand is fastened to the manipulator by a U-shaped clamp from the bottom of which a short rod extends. This rod is in turn screwed into a ball which is mounted in a socket through which another screw fits to clamp

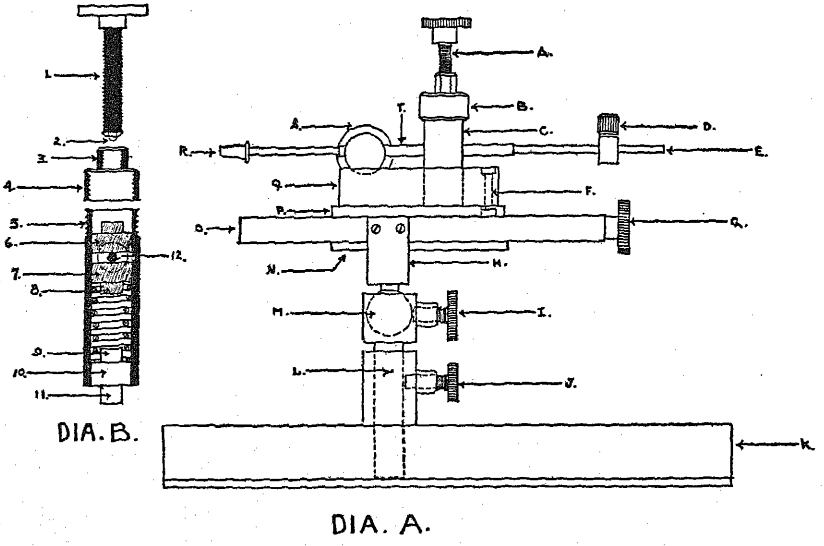

FIG. 1. A. DIAGRAMMATIC ENSEMBLE VIEW OF MICRO-MANIPULATOR. B. DETAIL OF MOVEMENT CYLINDER

Diagram A. *A*, up-and-down movement screw; *B*, threaded cap to receive screw; *C*, cylinder up-and-down movement; *D*, adjustable collar to act as a check; *E*, instrument holder tube; *F*, pivot on which the cylinder block turns; *G*, back-and-forth movement screw; *H*, u-shaped clamp for mounting stand to the micromanipulator; *I*, set screw for clamping ball in place; *J*, set screw for clamping rod in place; *K*, steel base; *L*, rod for raising the instrument up-and-down; *M*, ball for turning instrument to different angles; *N*, bottom bearing plate of back-and-forth movement; *O*, back and forth movement plate; *P*, upper bearing plate of back-and-forth movement; *Q*, block for mounting movement cylinders; *R*, Luer needle adaptor; *S*, side-to-side movement screw; *T*, guide-sleeve for instrument holder tube.

Diagram B. *1*, movement screw, threaded 80 threads to the inch; *2*, ball bearing to minimize friction; *3*, teat on the threaded cap to receive movement screw; *4*, cap threaded on inside to go on the threaded portion of cylinder; *5*, threaded portion of cylinder; *6*, upper bearing cylinder; *7*, lower bearing cylinder; *8*, teat on lower bearing to act as a seat for a spring; *9*, teat on plug around which spring is seated; *10*; plug; *11*; teat on lower surface of plug to mount the cylinder on a base; *12*, ball which bears the guide-sleeve.

the ball firmly in place. The bottom part of the socket is fastened to a rod which fits into a hollow pillar on the base of the stand. A set screw fitting through this pillar fastens the rod into position. Rubber is glued to the bottom of the steel base to prevent slipping of the instrument. Loosening the set screw in the pillar cylinder allows the manipulator to be raised or lowered to the desired height. Loosening the set screw in the socket allows the manipulator to be turned to any angle in relation to the stage of the microscope.

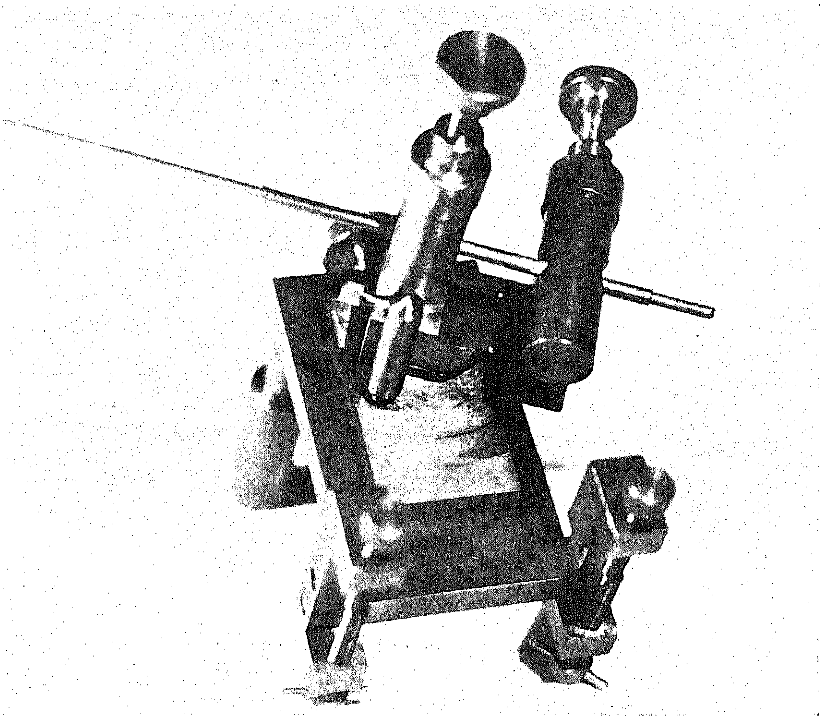

FIG. 2. SHOWING PIPETTE CHANGING MECHANISM SWUNG ON ITS AXIS

METHOD OF MOUNTING THE PIPETTES

Glass micro-pipettes and rods are used with this apparatus for single cell isolations. In place of the usual capillaries drawn directly from glass tubing, those used with this manipulator are drawn from capillary glass tubing, and are only one-fourth to three-eighths of an inch in length, from tip to base.

These pipettes are mounted in adaptors which consist of 14 gauge Luer slip needles cut off to ½-inch lengths, and bent almost to right angles ¼ inch from the end. A touch of glass cement is placed on the side of the pipette and it is slipped into the bent part of the needle. The cement effectively seals the pipette in place. The needle with the pipette is slipped over an adaptor soldered to the end of a length of brass tubing, the instrument holder. The instrument holder is held by a guide-sleeve, which

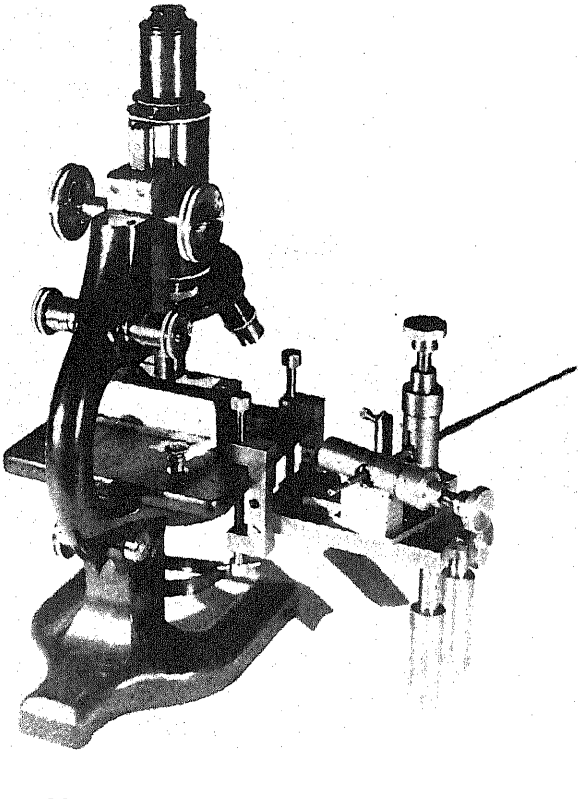

FIG. 3. SHOWING MICRO-MANIPULATOR IN POSITION ON MICROSCOPE STAGE WITH PIPETTE CHANGING MECHANISM SWUNG ON ITS AXIS

is inserted through the center of the two balls of the movement cylinder. An adjustable collar is then slipped over the back end of the instrument holder. This arrangement allows the pipette to be moved to and fro with the fingers and satisfies the requirements for a quick-acting coarse adjustment, to center the pipette. The adjustable collar acts as a stop to limit the distance the pipette can be pushed forward, and the base of the Luer needle adaptor acts as the check on the distance it can be pulled backward. Since the sleeve is close fitting and straight, there is very little deviation from plane and consequently adjustments may be made quickly and easily with the fingers instead of the special coarse adjustments found on some manipulators.

This method of mounting the pipettes provides shorter, stiffer instruments which are easy to make and mount.

USE OF THE MANIPULATOR IN SINGLE CELL ISOLATION

A moist chamber is set up in position on the stage of the microscope and the micro-manipulator placed in line and at the correct height in relation to it. A trial pipette is slipped into an adaptor. The instrument holder is pulled back as far as it will come, and the movement cylinders turned on their pivot so that the needle adaptor is facing the operator. The needle with the pipette is pushed on the adaptor and the instrument returned to normal position with the pipette in line with the opening of the moist chamber. The set screw in the adjustable collar is loosened and the collar pushed flush with the end of the guide-sleeve. The instrument holder tube is pushed forward with the fingers until the pipette can be seen with the low power lens. The tip of the pipette is centered in the field with the screw adjustment collar tightened. Once the pipette is centered it can be replaced with a sterile one, by lowering it away from the roof of the moist chamber, pulling it back as far as it will go, and then turning the movement cylinders on their pivot. Once the preliminary adjustments of centering the pipettes in the low power field have been made by hand, they need not be altered except slightly as the pipette is changed. Since the guide-sleeve is in a true line, there is no danger of breaking the delicate tip of the pipette and the entire procedure becomes easy and rapid.

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THE AMERICAN TYPE CULTURE COLLECTION

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The Culture Collection has been maintained for seven years by the John McCormick Institute for Infectious Diseases under the general direction of an incorporated committee of seven members, representing interested societies and the McCormick Institute.

The growth of the Collection in this time is shown in table 1.

TABLE 1

YEAR ENDING DECEMBER 15	1925	1926	1927	1928	1929	1930	1931
Cultures available	750	1,193	1,500	2,000	2,272	2,326	2,375
Orders filled	360	558	613	1,022	1,127	1,294	1,023
Cultures sent out	1,540	2,715	3,897	4,761	5,080	5,253	5,019

The funds through which the Collection has been maintained have been obtained from the sale of cultures, The Society of American Bacteriologists, and from a grant from the Rockefeller Foundation. The grant from the Rockefeller Foundation has expired and under the present economic conditions the Committee has been unable to obtain a renewal of this grant or to secure funds from other sources. It has been necessary to reduce expenses by decreasing the staff, reducing salaries and exercising other economies. This has made it necessary to reduce the Collection somewhat by eliminating duplicates and some of the cultures for which there is little call will be placed in the care of volunteer curators.

The price of cultures has been increased from one to two dollars. The committee has taken this action very reluctantly and as the alternative of abandoning the Collection. The income for the present year will be limited to $400 from the Bacteriological

Society, $400 interest on bonds in which surplus funds were invested, and receipts from the sale of cultures. If the latter item is equal to the average of the past two years, it will be possible to balance the budget without decreasing the usefulness of the Collection. In the early years of the Collection the sale of cultures was confined almost entirely to those used for teaching purposes. Its present wider usefulness is shown by the increasing call for cultures for research and industrial purposes. This has included the fungi, of which nearly 350 cultures were sold in the past year. Contact with special collections of bacteria, yeasts and other microörganisms greatly enlarges the variety of cultures the Collection is able to furnish its patrons.

FURTHER STUDIES UPON THE EFFECT OF VARIOUS CARBOHYDRATES ON PRODUCTION OF DIPHTHERIA TOXIN WITH SPECIAL REFERENCE TO ITS FLOCCULATING TITER AND FINAL pH

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INTRODUCTION

As early as 1896 Park and Williams (1896) recommended the use of glucose in small quantities as an aid in the production of toxic filtrates from *C. diphtheriae*. In contrast to Spronck (1895) they found, moreover, that the muscle sugar present in the infusion broth was not harmful to toxin production. T. Smith (1899) a short time afterwards, following a very complete study on the relation of glucose to the production of diphtheria toxin, reported that glucose added in small amounts to sugar-free meat infusion broth considerably enhanced the production of diphtheria toxin. Since this early work apparently little attention has been paid to the use of glucose in toxin production until the work of Wadsworth and Wheeler (1928). These authors found that glucose is essential for growth and toxin production in meat-infusion sugar-free peptone medium. Locke and Mann (1928) and Ramon (1929) have also only recently reported the use of glucose in the production of high-titered diphtheria toxin. Glucose in 0.2 per cent concentration is used routinely in the production of diphtheria toxin by the Division of Laboratories and Research of the New York State Department of Health. Earlier in the year we (Hazen and Heller) (1931) published a brief report of studies in which glucose and maltose had been used to enrich culture media for growth of *C. diphtheriae*. We found that the addition of either sugar to the culture medium definitely increased the Lf[1] unitage of the filtrates and that the single addition of a combination of the two sugars yielded filtrates of still higher potency within a period of only five days.

The present work is a further study of the effect of glucose and maltose on the production of diphtheria toxin together with a study of other carbohydrates fermentable by *C. diphtheriae*, such as dextrin and glycerol.[2] At the same time we have endeavored to correlate as far as possible the final pH of the filtrates with the Lf titer.

TECHNIQUE

The medium used in these experiments was made of bob veal infusion to which was added 2 per cent proteose peptone. Especial attention was paid to the quality and freshness of the veal as well as to the sterilization of the medium. The final pH of the medium was 7.8. The sugars we made up in 20 per cent solutions. Formerly they were heated in the Arnold for twenty minutes for three successive days; more recently it has been found more efficacious to sterilize the solutions by filtration through a Berkefeld candle. Only chemically pure sugars were used in this work. The Park-Williams No. 8 strain which was used throughout the work was maintained by transfer twice daily in the veal infusion broth.

The broth was inoculated with one large loopful of an actively growing pellicle. The cultures were grown in 300 cc. quantities in 1-liter flasks. The flasks (diagram No. 1) were specially designed so that frequent pH titrations and flocculation tests, if desired, could be carried out on the same specimen without disturbing the pellicle by manipulation of the flask.

Determinations of the M.L.D. were made on only a few of the filtrates. The Ramon flocculation test was carried out in the routine manner on the various filtrates.

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[1] The term is used in accordance with Glenny and Wallace's definition indicating "that amount of toxin (corresponding to one unit of a certain antitoxin) in that mixture which flocculates most rapidly when a series of mixtures of that toxin and antitoxin are set up in varying proportions and observed under constant conditions" (Topley and Wilson).

[2] Classed with the carbohydrates only because it is fermentable by *C. diphtheriae*.

EXPERIMENTAL WORK

The various carbohydrates (glucose, maltose, dextrin, and glycerol) were utilized as follows:

1. Small amounts of the single carbohydrate were added to the growing culture once or twice daily for a period of forty-eight to seventy-two hours, each addition of the sugar being preceded by pH titrations and adjustment of the culture fluid to pH 8.0. Control flasks with the same sugar concentrations, remaining unadjusted, were carried along at the same time.

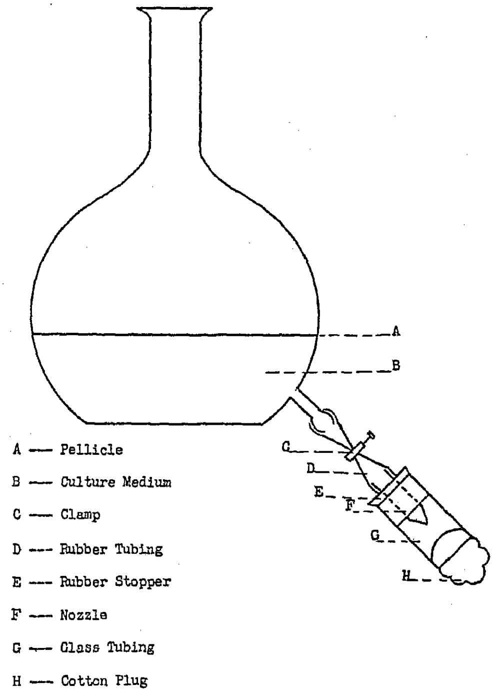


Diagram 1. Special Flask

2. The carbohydrate (glucose, maltose, dextrin, glycerol) was added to the medium before planting the pellicle with daily titration of the culture fluid.

3. A given amount of two carbohydrates in combination was added to the broth before planting the pellicle with daily adjustment of the culture fluid to pH 8.0. Control flasks of similar composition but unadjusted pH were run at the same time.

I. EFFECT OF FRACTIONAL ADDITION OF VARIOUS CARBOHYDRATES ON THE Lf TITER OF TOXIC FILTRATES OF C. DIPHTHERIAE

Semidaily additions of glucose in 0.15 per cent concentration to the growing filtrates with and without pH adjustment

Sterile 20 per cent glucose solution was added to six 1-liter flasks, containing 300 cc. of broth so as to yield a final concentration of 0.15 per cent. After preliminary incubation for sterility these flasks were divided into two groups, I and II. The first group, I, consisted of 3 flasks numbered 1, 2 and 3; the flasks in the second group were numbered 4, 5 and 6. Each of the six flasks was inoculated with one large loopful of a young, actively growing pellicle incubated at 37.5°C. Sixteen hours later 30 cc. of filtrate was withdrawn from flasks 1 and 4 for pH titration and Lf determinations. Flasks 1, 2 and 3 (group I) were then adjusted to a pH of 8.0 by adding the required amount of 50 per cent NaOH solution, the flasks in group II remaining unadjusted. Glucose solution was then added again to all six flasks to obtain 0.15 per cent concentration. In order to avoid too drastic a reduction of volume the experiment was arranged in such a manner that no more than 2 samples were drawn from the same flask during the whole test. Eight hours after the second addition of the sugar, flasks 1 and 4 were removed from the incubator and pH titrations and Lf determinations again made on the filtrates. The remaining flasks of group I were brought to pH 8.0, while the sugar concentrations, in all four flasks (2, 3, 5 and 6) were again reëstablished to 0.15 per cent. Sixteen hours later, specimens were again obtained from flasks 2 and 5 for pH titration and flocculation. Adjustment of pH of flasks 2 and 3 (group I) and addition of sugar to the four flasks, as previously described were again carried out. Eight hours later, flasks 2 and 5 were removed from the incubator and titration of pH and flocculation tests were made on their filtrates. The two last flasks, numbers 3 and 6, were treated as in the beginning. Sixteen hours later, titration of the pH and flocculation tests on samples from these flasks were made in the usual manner followed by adjustment of the pH of flask 3 and the final addition of glucose in a concentration of 0.15 per cent to the two flasks (3 and 6). Eight hours later, i.e., seventy-two hours after the beginning of the experiment, the last two flasks were removed from the incubator for a final pH titration, and flocculation of the filtrates.

The results of the pH titrations and the Lf values of the two groups of flasks are given in chart 1.

As can be seen from chart 1, there was a rapid and continued increase in toxin production during the seventy-two hours' incubation in the flasks (1 to 3) in which there had been an adjustment of the hydrogen-ion concentration. The final value obtained was 15 Lf per cubic centimeter with a pH of 8.7. In the flasks (4 to 6) receiving the same amount of glucose but remaining unadjusted there was a much slower accumulation of toxin so that at the end of the seventy-two hours only one-half the Lf value of the adjusted filtrate was reached. However, an Lf of 8.7 per cubic centimeter within seventy-two hours is not insignificant with a pH as low as 5.6. The control broth alone at this time yielded only 9 Lf per cubic centimeter with a pH of 8.2.

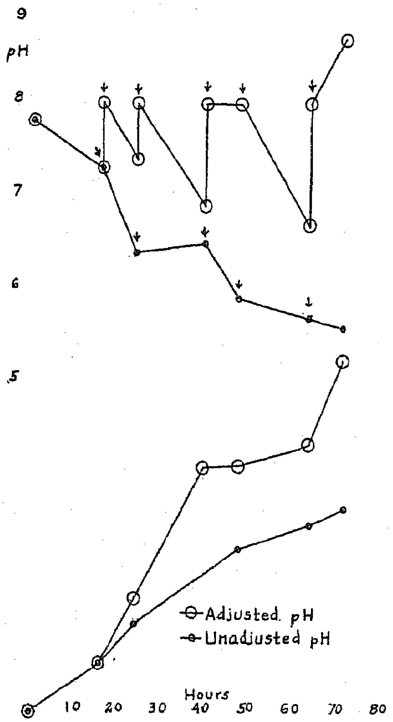
It is interesting to note here that the flocculating time of the unadjusted filtrate was occasionally 5 times as long as that of the adjusted filtrates.

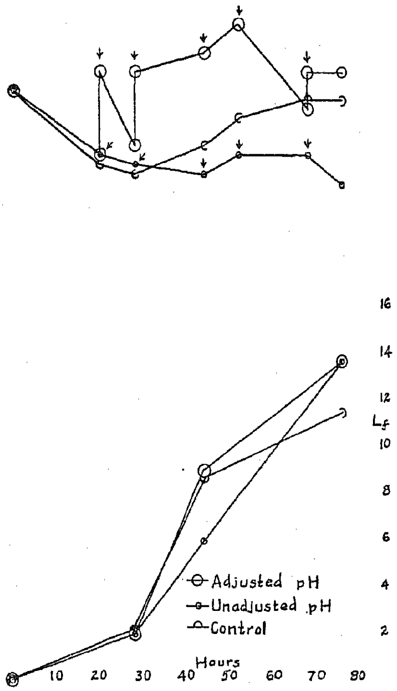
Semidaily additions of maltose in 0.15 per cent concentration to the growing culture filtrates with and without pH adjustment

The technique for this experiment is essentially the same as given above in detail except that maltose was substituted for glucose.

The results of the pH titrations and Lf determinations on the different filtrates are given in chart 2.

The Lf graphs of chart 2 show that there is an enhancement in toxin production when small amounts of maltose are frequently added to the growing cultures of *C. diphtheriae.* The chart would also seem to indicate that there is no advantage to be gained from an adjustment of the hydrogen-ion concentration of the culture medium at the time of adding the maltose, as was found





Arrow indicates addition of carbohydrate

CHART 1

CHART 2

CHART 1. EFFECT OF REPEATED ADDITIONS OF GLUCOSE (0.15 PER CENT CONCENTRATION) ON Lf AND pH OF DIPHTHERIA TOXIN

CHART 2. EFFECT OF REPEATED ADDITIONS OF MALTOSE (0.15 PER CENT CONCENTRATION) ON Lf AND pH OF DIPHTHERIA TOXIN

necessary in the experiment with glucose. While the final pH of the unadjusted filtrate was 6.8, of the adjusted filtrate 8.0, and the control 7.7, the final Lf of both of the maltose filtrates was 13.7 per cubic centimeter contrasted with an Lf of 11.5 per cubic centimeter for the control.

Semidaily additions of dextrin in 0.15 per cent concentration to the growing culture filtrates with and without pH adjustment

The experimental procedure here is the same as that given for the first experiment except that dextrin was substituted for glucose.

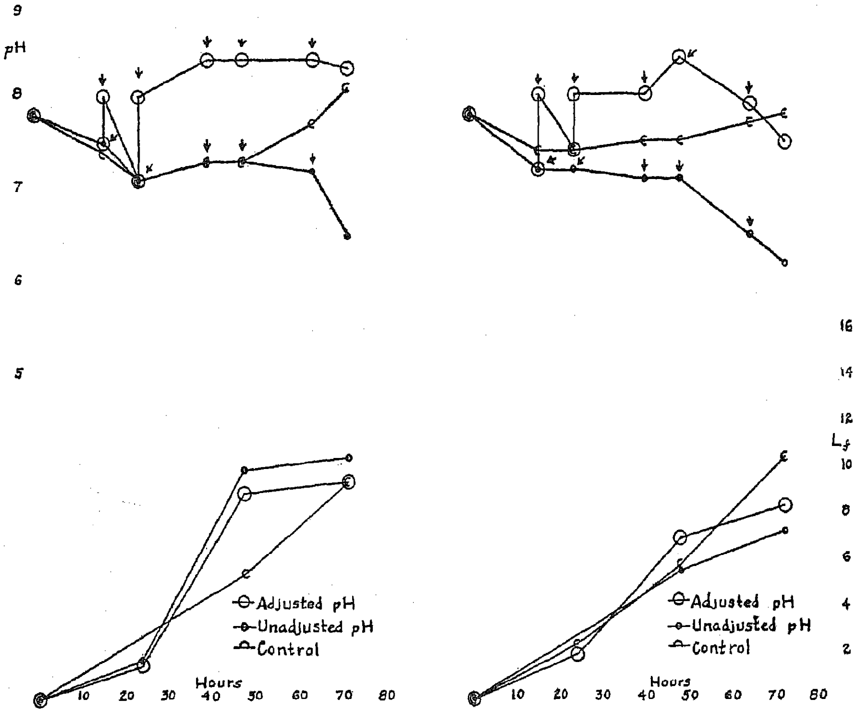
The results obtained from this experiment are given in chart 3. The Lf graphs in chart 3 show that frequent additions of dextrin to growing filtrates with adjusted or unadjusted pH do not enhance toxin production materially, at least not during a period of seventy-two hours. The pH curve of the unadjusted filtrate for the first forty-eight hours suggests that dextrin is utilized only very slowly by *C. diphtheriae.* The final pH of the adjusted filtrate was 8.3, that of the unadjusted 6.5, while that of the control broth was 8.1. The Lf titers of the three filtrates differed only slightly, with an Lf of 9.5 per cubic centimeter for the unadjusted filtrate and the control and 10.5 Lf per cubic centimeter for the adjusted filtrate.

Semidaily additions of glycerol in 0.15 per cent concentration to the growing culture filtrates with and without pH adjustment

The details of such an experiment have been fully described in the glucose protocol.

Chart 4 would suggest that frequent additions of glycerol actually inhibited toxin production. The adjusted filtrate showed a final Lf titer of 8.4 per cubic centimeter; the unadjusted filtrate 7.3 while the broth control had a final titer of 10.5 Lf per cubic centimeter. The pH graphs of the glycerol filtrates show a final pH of 7.5 for the adjusted filtrates, 6.2 for the unadjusted filtrate and a final pH of 7.7 for the control broth.

In summing up our observations, it would seem that semidaily additions of glucose to growing diphtheria cultures considerably enhance the Lf titer of the toxin, provided the pH of the filtrates is adjusted at the time of adding the sugar. Fractional additions of maltose to the growing cultures were likewise found to increase the flocculating titer of the filtrates. However, adjustment of the pH of the culture fluid at the time of adding maltose was found to be unnecessary. Frequent additions of small amounts of dextrin to the cultures, with or without adjustment of pH, were not found to affect the Lf titer of the filtrates. Finally, frequent additions of glycerol to the growing cultures were actually deleterious to the production of toxin.



Arrow indicates addition of carbohydrate

CHART 3 CHART 4

CHART 3. EFFECT OF REPEATED ADDITIONS OF DEXTRIN (0.15 PER CENT CONCENTRATION) ON Lf AND pH OF DIPHTHERIA TOXIN

CHART 4. EFFECT OF REPEATED ADDITIONS OF GLYCERINE (0.15 PER CENT CONCENTRATION) ON Lf AND pH OF DIPHTHERIA TOXIN

II. EFFECT OF A SINGLE ADDITION OF GLUCOSE, MALTOSE, DEXTRIN OR GLYCEROL TO GROWING CULTURES OF C. DIPHTHERIAE ON THE Lf TITER OF THE TOXIN

Effect of 1 per cent sugar concentration (glucose, maltose)

Eight 1-liter flasks, each containing 300 cc. of broth, were divided into two sets of four each. To one-half of the flasks 1 per cent glucose was added, the other half receiving 1 per cent maltose. The 8 flasks, together with a plain broth control flask were then inoculated and, after twenty-four hours' incubation, a pH titration as well as an Lf titration was made on all filtrates. Two flasks from each series were then adjusted to a pH of 8.0, this procedure being repeated for five consecutive days. The results of the daily titrations are given in chart 5.

The results of this experiment show that a single addition of 1 per cent glucose inhibits toxin production in so far as can be determined by the flocculation test, for at no time were we able to obtain flocculation with any of these glucose filtrates, adjusted or unadjusted. Growth in the flasks containing glucose was poor at the end of twenty-four hours. Although the pH never fell below 6.0 the rapid accumulation of acid within the first twenty-four hours of growth was apparently sufficient to handicap the organisms for further toxin production, to such an extent that later adjustment of the pH to 8.0 had no effect.

Quite opposite results were obtained with the maltose. With both adjusted and unadjusted filtrates, there was a continuous and abundant production of toxin, the final Lf attaining 17.5 per cubic centimeter while with the control broth only an Lf of 9 per cubic centimeter was obtained. Here again, adjustment of the pH of the culture was of no value. At no time did the pH of the unadjusted filtrate fall below 6.6. It is interesting to note that these filtrates were found to contain between 1000 to 1200 M.L.D.'s per cubic centimeter when tested subcutaneously on guinea pigs of 250 grams. Further experiments with maltose demonstrated that as much as 3 per cent could be added at one time to the culture fluid without producing a deleterious effect.

Effect of 0.45 per cent carbohydrate concentration

Twelve flasks containing 300 cc. each of broth were divided into four sets of three flasks each. To each set of flasks there was added 0.45 per cent concentration of glucose, maltose, dextrin and glycerol respectively. The entire series, together with a control flask of plain broth, was inoculated with a fresh pellicle of *C. diphtheriae* and daily titrations of the pH and Lf determinations of the different filtrates were carried out for five days. The results obtained in this experiment are given in chart 6.

As may be seen from chart 6 a single addition of 0.45 per cent concentration of carbohydrate to the culture fluid at the time of planting the pellicles is of doubtful value with the majority of the carbohydrates tested. Only the filtrates containing maltose in 0.45 per cent concentration showed a higher titered toxin than

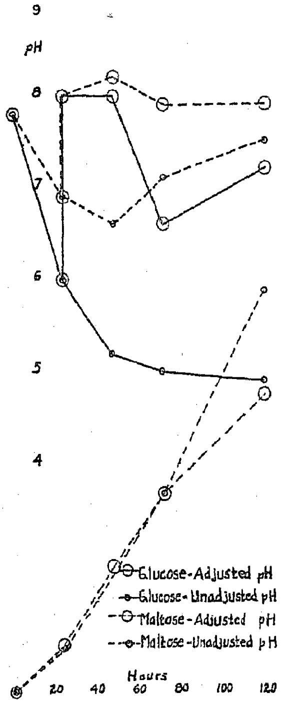


CHART 5

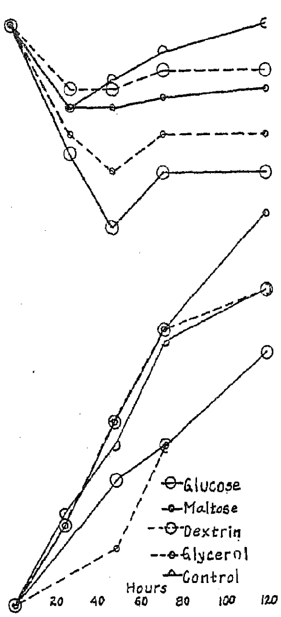


CHART 6

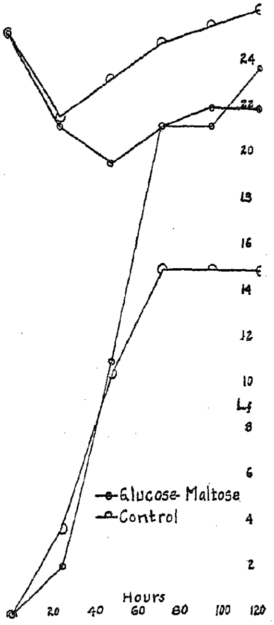


CHART 7

CHART 5. EFFECT OF A SINGLE ADDITION OF CARBOHYDRATE (1 PER CENT CONCENTRATION) ON Lf AND pH OF DIPHTHERIA TOXIN

CHART 6. EFFECT OF A SINGLE ADDITION OF CARBOHYDRATE (0.45 PER CENT CONCENTRATION) ON Lf AND pH OF DIPHTHERIA TOXIN

CHART 7. EFFECT OF A SINGLE ADDITION OF GLUCOSE (0.15 PER CENT CONCENTRATION) AND MALTOSE (0.3 PER CENT CONCENTRATION) ON Lf and pH OF DIPHTHERIA TOXIN the control in five days. While the filtrates containing dextrin in the same concentration had an Lf titer equal to that of the control, the Lf titer of the filtrates containing 0.45 glucose concentration was less than that of the control. We were unable to obtain a flocculation phenomenon with the filtrate containing 0.45 per cent concentration of glycerol after the third day of incubation when the observed Lf titer was 7 per cubic centimeter. The marked inhibition in toxin production in the glycerol-containing medium can hardly be explained on the basis of excessive acidity for the lowest pH reached during the five days of incubation was 6.2.

TABLE 1

Effect of a single addition of carbohydrate (0.15 per cent concentration) on Lf and pH of Diphtheria toxin

CONCENTRATION OF CARBOHYDRATE IN CULTURE MEDIUM	FINAL pH (5 DAYS' INCUBATION)	Lf PER CUBIC CENTIMETER (5 DAYS' INCUBATION)
0.15 per cent glucose	7.7	9.0
0.15 per cent maltose	7.8	7.5
0.15 per cent dextrin	7.7	5.0
0.15 per cent glycerol	8.0	8.5
Plain broth control	7.0	6.0

Effect of 0.15 per cent carbohydrate concentration

The results of the final titration of the hydrogen-ion concentration of the filtrates and the final Lf values for the different filtrates are given in table 1.

It will be seen from table 1 that a single addition of maltose or glycerol in 0.15 per cent concentration to the medium at the time of planting the pellicles increased the yield of toxin within five days somewhat over that of the control, while dextrin in the same amount apparently had no effect on toxin production. Glucose on the other hand in similar amounts enhanced the Lf titer of the filtrates considerably. While a small addition of glucose to the culture fluid appears to favor toxin production more than maltose in equivalent amounts, this is not true for larger additions of the two sugars.

Addition of a given amount of two carbohydrates in combination to the culture fluid

We next attempted to determine the effect on toxin production of combining the two sugars. Since adjustment of the pH of the culture filtrates is laborious as well as impractical for routine work, we thought that by adding glucose and maltose simultaneously in varying amounts it might be possible to obtain a combination which would not only augment the yield of toxin but at the same time prevent the drop in pH which accompanies the fermentation of glucose by *C. diphtheriae*.

The final results obtained from the single addition of the combined sugars are shown in table 2.

TABLE 2

Effect of a given amount of two carbohydrates in combination on Lf and pH of diphtheria toxin

FLASKS	CONCENTRATION OF GLUCOSE IN MEDIUM	CONCENTRATION OF MALTOSE IN MEDIUM	FINAL pH (5 DAYS)	M.L.D.	Lf PER CUBIC CENTIMETER
	per cent	*per cent*			
Control			7.7	770	9.6
A	0.15	0.15	7.4	1,400	17+
B	0.15	0.3	7.1	2,100	26.3
C	0.3	0.15	6.9	1,400	17+
C	0.3	0.3	6.4	1,260	15.7

It will be noted from table 2 that in every flask to which the two carbohydrates were added there was an increase in the Lf titer of the filtrates when compared with that of the plain broth control. Since a combination of 0.15 per cent glucose and 0.3 per cent maltose yielded filtrates of the highest Lf value, with a final pH of 7.1 at the end of five days, we used the two sugars in these amounts in further experiments and obtained uniformly most satisfactory results. Chart 7 shows a typical experiment.

Our experience in the past year with a variety of toxins produced by various carbohydrates has in every instance indicated the best results with the use of this combination. During the winter months, when diphtheria toxin is more difficult to produce in high titer we obtained with this method within five days filtrates of 15, 17 and 21 Lf per cubic centimeter. Povitsky (1931) reports that she was unable to obtain in a volume of 600 cc. a higher titered toxin by addition of the combined sugars (glucose and maltose) than was obtained by the routine method (0.15 per cent concentration of glucose) and that the pH of those filtrates made them impractical for toxoid purposes. We have been able to produce toxoids from such filtrates (pH 7.1) within the usual time limit for toxoid production. Such toxoids, moreover, flocculated within twenty-five to forty-five minutes with the same antitoxin which we used with the original toxin. Dr. Florence Stone in this Department has obtained with this method in a volume of 100 cc. within five days filtrates of 30 or more Lf per cubic centimeter. With the proper adjustment of the initial pH of the medium and sterilization of the sugar by Berkefeld filtration it should be possible to obtain satisfactory filtrates with a volume of 600 cc. by this method.

SUMMARY

Studies on the daily changes in pH and toxin production in media containing glucose, maltose, dextrin and glycerol after inoculation with *C. diphtheriae* have yielded the following results:

1. Glucose added twice daily in concentration of 0.15 per cent to the growing cultures during the period of seventy-two hours' incubation definitely increased the Lf titer of the filtrates, provided the medium was alkalinized at the time the sugar was added. Similar additions of maltose in 0.15 per cent concentration likewise increased the Lf titer of the filtrates without resorting to pH adjustment. Dextrin or glycerol under similar experimental conditions failed to increase the yield in Lf units per cubic centimeter.

2. Glucose added only once to the culture broth at time of inoculation in 0.15 per cent concentration stimulated a more abundant toxin production within five days than was obtained with glucose free broth. A fairly potent toxin was also harvested with glucose added in 0.45 per cent concentration to the culture fluid at time of inoculation, but the titer did not equal that of

the control broth. Larger amounts of glucose inhibited toxin production. Smaller amounts of maltose added at the time of seeding likewise increased the Lf unitage of diphtheria toxin, excellent and equally good results being obtained with 0.45 and 1 per cent concentrations of this sugar. A single addition of dextrin in the amounts stated above did not increase the Lf value while glycerol added in 0.15 per cent concentration definitely enhanced toxin production. Lower values were obtained with larger quantities of glycerol.

3. With a combination of glucose and maltose (0.15 and 0.3 per cent concentration) added to the culture fluid at time of inoculation, filtrates containing as high as 26.3 Lf per cubic centimeter were obtained within five days. This combination of sugars proved to be more efficacious than any other method tried.

The final reactions at which highly potent filtrates were harvested were not found to lie within the commonly accepted range of pH 7.8 to 8.25 (Bunker (1919)). The strongest toxins had a final pH of 7.1. Daily titrations of pH moreover showed that the reactions never approached the critical zone laid down by Bunker. This author found potent toxins only in cultures with a final pH of 7.8 to 8.20. Our observations therefore agree with those of Hartley and Hartley (1922) who also reported high toxins with variations in pH from 7.5 to 8.9. Since our work was based on toxin production with a comparatively small volume (300 cc.) we have no way of telling whether different results may be obtained with larger quantities.

CONCLUSIONS

1. Carbohydrates are essential for the production of toxic filtrates of *C. diphtheriae* possessing highest potency.
2. Glucose in small amounts is definitely superior to maltose, dextrin or glycerol as an aid to toxin production.
3. Larger amounts of maltose than it is possible to use in the case of glucose without adjustment of pH increase the Lf titer of the filtrates of *C. diphtheriae*.
4. Dextrin in the amounts tested has no demonstrable effect on toxin production.

5. Glycerol in 0.15 per cent concentration increased toxin production while larger quantities were inhibitory.

6. Filtrates of the highest Lf were obtained with a combination of glucose in 0.15 per cent concentration and maltose in 0.3 per cent concentration.

7. The final pH of the filtrate does not necessarily seem to be an infallible index as to the best time of harvesting the toxin. It would seem therefore that the toxicity of a given filtrate is largely independent of the particular final pH reaction at the end of incubation.

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SPIRELLA SP.? ISOLATED FROM THE GUINEA PIG

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There is a large group of spiral organisms, isolated from normal and diseased animals and man, which seem to have no pathogenic action by themselves. They vary so much in morphology and cultural requirements that it is almost impossible to classify them. They are sometimes referred to as the muco-cutaneous spirochetes or spirilla and are generally classified as those of the mouth, and those of the intestines, although it is possible that the same species may be in both groups (Macfie (1917); Parr (1923)).

The question of the pathogenicity of these organisms is not definitely settled. They seem to be more numerous in diseased conditions of the gums or intestinal tract than in normal conditions (DeLamare (1924); Macfie (1917); Thompson and Thompson (1913–14)). Most workers have found it very difficult to infect animals experimentally with these organisms, but occasionally, if the animal's resistance is lowered, or if newborn animals are used, they may become infected (Hogue (1922); Kasai and Kobayashi (1919); Parr (1923); Sanarelli (1927)). The resemblance of some of these organisms to the definitely pathogenic forms raises questions which must be investigated by any one dealing with spirochetal infections, particularly in this instance, by those studying rat-bite fever.

MATERIAL

The organism described in this paper was isolated from some guinea pig infusion medium which was used in an attempt to cultivate the *Spirochaeta morsus-muris* of rat-bite fever. The medium was made as follows:

The intestines were removed from one apparently normal male guinea pig. The stomach was left in the body. The whole animal was then ground in a meat grinder and mixed with an equal quantity of distilled water. This mixture stood in the cold room for twenty hours and was stirred occasionally. The infusion was then squeezed through gauze and centrifuged at 3000 r.p.m. for half an hour to remove the sediment and fat. The thick fluid was poured off and filtered slowly through paper. Part of this filtration took place at room temperature, but it was so slow that it was left for completion in the ice box overnight. The next morning some of the medium had frozen on the paper. The filtrate was adjusted to pH 7.2 to 7.4, and refiltered through a Berkefeld V candle, and tubed in eight 10 cc. lots and one 20 cc. lot. Seven of the tubes were inoculated with heart's blood from a guinea pig infected with rat-bite fever, and three of them were inoculated also with tissue from another guinea pig infected with rat-bite fever. The tubes were incubated at 37.5°C. aerobically and anaerobically as follows:

Tube 1. Heart's blood, incubated aerobically.
Tube 2 (0.1 per cent Douglas agar). Heart's blood, incubated aerobically.
Tube 3 (covered with vaseline). Heart's blood, incubated aerobically.
Tube 4 (20 cc.). Heart's blood + heart, incubated aerobically.
Tube 5. Heart's blood + spleen, incubated aerobically.
Tube 6. Heart's blood + kidney, incubated anaerobically.
Tube 7 (0.1 per cent Douglas agar). Heart's blood, incubated anaerobically.

Two uninoculated tubes were kept in reserve for subcultures. Unfortunately there was so little of the medium that none was incubated as a control.

After three days the medium in tube 2 was broken up into small clumps near the surface and had turned a dull brown. When these clumps were examined with a darkfield microscope, they showed many small darting organisms which had the appearance of a closed safety pin. When the motility became slower, each organism was found to have one or two spiral curves. Except for the organ cultures in which there were Gram-negative bacilli, all the other cultures were sterile. Because the organism showed certain morphological resemblances to a Boston strain of *Spirochaeta morsus-muris,* it was called the B_1 organism.

MORPHOLOGY

After numerous subcultures the organism shows a more definite spiral form than when first isolated. In films from a seventy-two-hour culture stained with Wright's stain, it averages 2μ to 3μ in length and 0.3μ in width with about one curve per μ. The ends taper into fine points. The smallest forms are S-shaped and only 1μ to 1.5μ long and 0.2μ wide. In old cultures there may be long forms with eight or more spiral curves. Films made from cultures more than seven days old show fewer spiral organisms and many coccoid bodies which have a diameter of about 0.3μ (fig. 1). Occasionally these round forms may be seen attached to the end of a spiral organism, or within the organism (fig. 3). They seem to be products of degeneration of the culture, because they increase in number as the culture grows older and they are not found in uninoculated medium which has been incubated for some time. Sanarelli (1927) described similar forms in cultures of an intestinal spirochete which he isolated. He believed that, when transferred to fresh medium, these coccoid bodies would develop into typical spirochetes. Except in the case of filtrates, transfers from an old B_1 culture have been successful only if some active spirochetes could be found by darkfield examination. Barred and swollen organisms may also be found in very old cultures.

The young organism has single bipolar flagella which give it a quick darting motion like that of the cholera vibrio. The flagella can be seen only in stained preparations (fig. 4). The older organism has a slow corkscrew-like motion or occasionally a violent lashing motion, turning over and over in place.

STAINING

The organism is Gram-negative but very poorly stained by that method. It is easily stained by the ordinary methods used for spirochetes, such as Wright's, Fontana-Tribondeau's (see Robertson (1930)), Giemsa's or Tunnicliff's (1921) methods, and also by gentian violet, methyl violet, carbol crystal violet, or carbol fuchsin. The flagella can be stained by Fontana-Tribondeau's method or by Plimmer's and Paine's stain (1921). Occasionally, Wright's stain or methyl violet shows a fine filament at each end of the organism (fig. 3).

REPRODUCTION

The organism multiplies by transverse division. In stained films from a seventy-two-hour culture, there are many long forms with four, six, or eight spiral curves which seem to be divided into sections of two curves each joined by a fine filament (fig. 2). Two of these sections may be almost at right angles to each other. In fresh preparations examined under the darkfield microscope, no filaments can be seen, but often two short organisms seem to be dragging each other back and forth across the field.

CULTIVATION

The organism grows best in a semi-solid nutrient medium such as Douglas broth plus 0.1 per cent agar. I have generally used a Douglas broth (pH 7.2–7.4) which has been boiled only once, and has been sterilized by filtration through a Berkefeld N candle. A transfer of one loopful of an actively growing culture into this medium will give growth in about a week. The usual inoculum is three or four drops from a capillary pipette. The tubes are covered with rubber caps to prevent evaporation. In twenty-four to forty-eight hours, there is a cloudy ring of growth near the surface of the medium. This growth may be in the upper liquid zone or may extend into the agar giving it a "fuzzy" appearance. Darkfield examination of the culture at this time shows many short darting organisms and very few coccoid bodies. After a week there is a great decrease in the number of active organisms, and the cultures generally die out in two or three weeks; but active organisms have been seen in cultures eighty-one days old. As the culture grows older a thin pellicle with a metallic or irridescent tint forms on top, and the agar near the surface breaks up into fine clumps. Cystine, glutathione, thioglycollic acid, and ethyl-

ethylxanthate, added to this Douglas medium in a concentration of 0.1 per cent, seem to promote more rapid growth. No odor is produced by the culture.

The organism has never grown in human, horse, rabbit or guinea pig serum, ascitic fluid or 2 per cent peptone water, whether these media were fluid or semi-solid. But maltose, glucose, lactose or sucrose added to semi-solid peptone water in a concentration of 1 per cent makes it a very suitable medium. The sugars are not fermented.

In 15 per cent gelatin incubated at 37.5°C. the organism grows in tiny flakes which are suspended in the medium for four or five days and then sink to the bottom. The growth is slow but the culture remains active for several weeks. There is no liquefaction.

Growth in fluid medium is more difficult to obtain, but a large transfer from an actively growing culture into the special Douglas broth described above generally produces a faint turbidity in two or three days. The growth is never very heavy and remains evenly distributed.

Repeated attempts have been made to grow the organism on solid media. Douglas agar, 2 per cent plain agar, Douglas agar to which 20 per cent rabbit serum has been added, rabbit's blood agar, prune agar, glycerol egg slopes, 1 per cent glucose agar, ascitic fluid agar, all proved unsuitable when inoculated from cultures grown on Dogulas semi-solid medium. Slopes of 5 per cent rabbit blood agar containing 1 per cent cystine and 1 per cent glucose give good growth in two or three days. The growth starts in the water of condensation near the butt, and occasionally forms in colonies on the slope. These colonies are tiny, round, raised and colorless, at first. Gradually they become a greyish brown color and increase in size up to about 1 mm. They may be dull and slimy or have an irridescent tint on the surface. The whole medium becomes an olive green color. Films made from growth on this medium show organisms which are almost straight, but when they are transferred to semi-solid media they return to a typical spiral form. Transfers from one of these slopes to *moist* slopes of 1 per cent glucose or maltose agar, glucose-ascitic agar, or Russell's double sugar agar slopes, give similar colonies, but the growth is slower and less profuse.

The organism grows best under aerobic conditions. If the culture is covered with vaseline, it may live as long as ninety days. In one case, when a mold grew over the top of the culture, the spiral organism remained alive for forty-two days.

RESISTANCE

Growth will not take place at room temperature, although a growing culture will remain alive for a day or two after it has been removed from the incubator. One culture incubated at 30°C. showed no growth after two days. It was then transferred to 37.5°C. and grew rapidly in twenty-four hours. In spite of the fact that the original medium was kept for three days in the cold room, repeated attempts to subculture from a culture kept there for twenty-four hours have failed. Transfers from cultures heated at 58°C. for ten minutes have also been unsuccessful.

Experiments have been carried out to determine the effect of bile salts on the organism. To 0.5 cc. of 15 per cent sodium taurocholate, and to 0.5 cc. of ox bile, 10 drops of a fluid culture three days old were added. The suspension was so thin that five more drops of a semi-solid culture were added. Particular care was taken to avoid the clumps, but in the latter case it was impossible to avoid them all. These mixtures were examined with the darkfield microscope and then incubated for one hour in a 37.5°C. water bath. They were examined again at the end of the hour. The sodium taurocholate had very little effect on the organisms except possibly to reduce motility. The bile mixture showed decidedly fewer organisms, and only one or two motile ones in a darkfield preparation. This experiment was repeated with the same results, which do not agree with the effect of bile salts on most spirochetes. It is possible that the small clumps were too impenetrable.

FILTRATION

All the growth from the surface of seven cultures, old and young, was transferred to a flask of 25 cc. Douglas broth, which had been inoculated with a culture of *B. prodigiosus* four hours earlier. The mixture was filtered through a Berkefeld N candle under a pressure of 25 cm. Hg in five minutes. The filtrate was tubed, made semi-solid by the addition of 0.1 per cent agar, and incubated at 37.5°C. After three days, there was a growth of the B_1 organism in all the tubes and the filtrate was free from bacilli. Cultures filtered through a Berkefeld W candle were sterile.

ANIMAL EXPERIMENTS

Two white mice were inoculated with 0.4 cc. of a four-day active culture. One was inoculated subcutaneously and the other intraperitoneally. Neither mouse ever showed the organism in its blood, nor showed any signs of illness. The one which had been inoculated subcutaneously died after eighty-nine days. No spiral organisms were found in the intestinal contents, the liver or spleen, either by direct examination or by culture. The other mouse died ninety days after it had been inoculated. A spiral organism morphologically identical to the B_1 organism was found in the intestines but all attempts to cultivate it failed.

Two guinea pigs were inoculated subcutaneously and intracutaneously with the organism, one with 0.6 cc. of the original culture when it was five days old, the other with 0.6 cc. of a second subculture which was twelve days old. Neither guinea pig showed any local lesions; they never became sick, and their blood never showed the organisms. One was killed after ninety days, the other after forty-three days. The stomach contents, liver, spleen, and intestines were carefully examined, but no spiral organism was found. The stomach contents of three guinea pigs which had died of rat-bite fever were also examined, but no spiral organisms were found.

A rabbit was injected intravenously with 2 cc. of a forty-eight-hour growth from the supernatant fluid of a semi-solid culture. Although some of the culture went into the tissues around the ear vein, there was never any local lesion, and the rabbit showed no signs of illness. Nine weeks later the same rabbit was injected intravenously with 5 cc. of a twenty-four hour broth culture. This injection was repeated a week later and the serum was tested for agglutinins against the B_1 organism. In dilutions of 1:20, and 1:40, it agglutinated the organism, but not in a dilution of 1:2 or undiluted. After two more injections, the serum agglutinated the organism weakly in low dilutions, strongly in dilutions of 1:20 and 1:40 and moderately in 1:160. No higher dilution has been tested.

Tests were made with this agglutinating serum to determine whether or not it had protective properties against infection with the spirochete of rat-bite fever. The serum, in dilutions of 1:10 and 1:20, was mixed with an equal quantity of defibrinated blood containing *Spirochaeta morsus-muris* from a guinea pig infected with rat-bite fever. After standing one hour in the dark at room temperature, 0.2 cc. of each mixture was injected intracutaneously into a normal guinea pig. After four days both sites of inoculation were inflamed and swollen, while there was no inflammation at the site of inoculation of a mixture of the same blood and an equal quantity of serum known to contain antibodies for the *Spirochaeta morsus-muris*. These lesions progressed and were found to contain the spirochete of rat-bite fever. Therefore the serum agglutinating the B_1 organism had no immune antibodies for *Spirochaeta morsus-muris*.

SUMMARY

A spiral organism resembling *Spirochaeta morsus-muris* has been isolated in pure culture from a guinea pig. Its exact source is not known.

The organism is Gram-negative, short, with regular curves, pointed ends and bipolar flagella. Numerous granules and coccoid bodies appear in older cultures.

The organism multiples by transverse division.

The organism does not resist the temperature of 58°C. for ten minutes, or of 2°C. for twenty-four hours. It lives for twenty-four or forty-eight hours but does not multiply at room temperature or at 30°C. It grows well at 37.5°C.

Bile salts do not dissolve it completely in one hour.

The organism grows best on a semi-solid nutrient medium such as Douglas broth plus 0.1 per cent agar. If a large inoculum is used it will grow on moist agar slants containing sugars, and in fluid media. Sugars are not fermented. The organism does not grow in human, horse, rabbit or guinea pig serum, or ascitic fluid.

Cultures of the organism may be filtered through a Berkefeld N candle, but not through a W candle.

The organism is apparently non-pathogenic to mice, guinea pigs and rabbits.

Agglutinins active against this B_1 strain of spiral organisms were produced by intravenous inoculation of a rabbit with living cultures. This antiserum had no protective properties against infection with *Spirochaeta morsus-muris*.

CONCLUSION

The organism described in this paper probably belongs to the muco-cutaneous group of spirochaetes, or spiral organisms. Although the intestines had been removed from the guinea pig before making the medium, the organism might have been in the stomach or mouth, or even in the blood. As the descriptions of organisms of this group are incomplete at present, it is impossible to identify this organism with any previously described strain. It resembles most closely the "Spirochetè caecal," described by Sanarelli (1927). Since it has morphological characteristics of both spirochetes and spirilla, it might be classified in the genus Spirella (Duboscq, and Lebailly, 1912).

These difficulties of classification indicate the need of further investigations to establish the characteristics and limits of this group of spiral organisms.

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PLATE 1

Reproductions of photomicrographs, ×1000 magnification

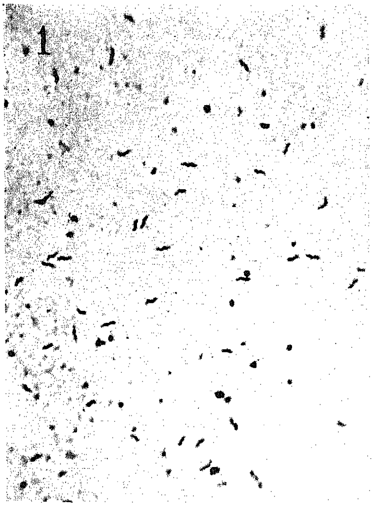
FIG. 1. Eight-day growth from the original culture, stained with Tunnicliff's stain. Note coccoid bodies.

FIG. 2. Five-day growth from fortieth subculture. Stained with methyl violet. Note pleomorphic forms with thickened ends and dividing form in the center at the bottom of the picture.

FIG. 3. Long form from the same culture. Stained with methyl violet. Note granule in the center of the organism and long tapering filaments.

FIG. 4. Film from three-day growth of thirty-eighth subculture stained with Plimmer's and Paine's flagella stain. A single flagellum shown at each end of the organism.

The prints of figures 2, 3 and 4 were retouched and rephotographed to increase the contrast. Details in the negatives are sharper than they are in these reproductions.



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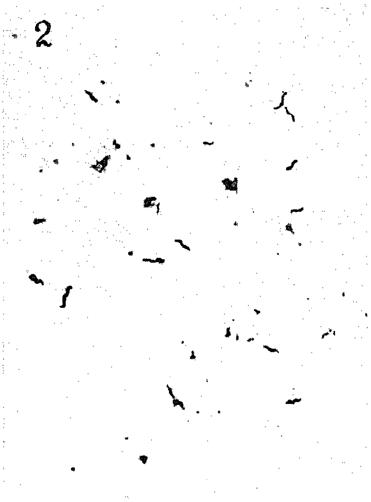

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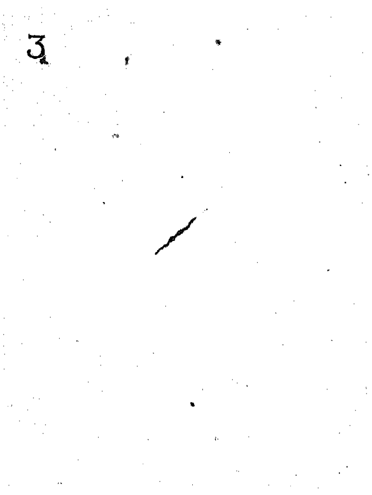

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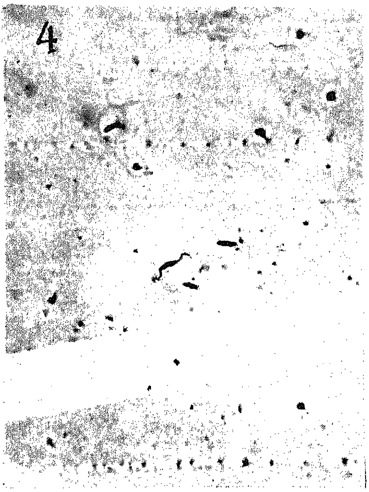

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(Henrietta S. Rhees: Spirella Sp.? isolated from guinea pig)

STUDIES ON ANTIBODIES

IV. THE CARBOHYDRATE CONTENT AS A CRITERION OF THE SUITABILITY OF A BROTH FOR THE CULTURE OF PNEUMOCOCCI

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INTRODUCTION

In studying the efficacy of antipneumococcus preparations, the properties of the pneumococci used in the tests with the antibody are obviously of paramount importance. Two factors appear significant in the consideration of broth cultures of pneumococci: the rate of growth and the virulence of the culture.

The acidity of broths as shown by the pH has been studied extensively and conditions for the most satisfactory rate of growth determined. Avery and Cullen (1919) using "glucose broths" containing 0.4 to 2.0 per cent glucose and also plain meat infusion broths found the optimum pH for growth at pH 7.8. With broths of an initial pH of 7.8 and more than 0.4 per cent glucose, growth ceased at pH 5.0; when no carbohydrate was added to the meat broth, growth ceased at about pH 7.0. They used sugar-free broth plus known amounts of glucose or other carbohydrate, but reported no carbohydrate estimations of the broth after different periods of growth of the pneumococcus. The different types of pneumococci, so far as tested, behaved alike. Dernby and Avery (1918) also found the growth optimum at pH 7.8, with pH 7.0 and 8.3 as the limits for growth. Cullen and Chesney (1918) considered that acid production with change in pH of medium paralleled the change in rate of growth, until at pH 7.0 growth ceased. They considered, however, that increase in acidity was not the sole cause of cessation of growth. Reference to the work of Wright (1929), may also be made in this connection.

Dr. Grund of the Research Laboratory of the Department of Health of New York City, with meat infusion broth media to which no carbohydrate was added, found most satisfactory growths at pH 7.4 to 7.6 (private communication).

It is generally accepted that animal passage increases or maintains the virulence of organisms and that in broth media virulence is diminished; the addition of certain body fluids to culture media has been found to aid in maintaining or even in increasing virulence at times. The experimental difficulties involved in making an accurate measure of virulence and the variation in the chemical constituents of broth media add to the uncertainties of the conclusions derived from such studies. The lack of accurate knowledge of the properties of such broth media and the possible variations of growth of organisms in different lots of media, even of those prepared apparently in the same way, is evident to any one who has worked with these materials. The more exact determination and adjustment of the acidities of media by tests for hydrogen ion concentrations marked a great advance in their preparation and use.

The results obtained in the study of (1) the initial carbohydrate contents of beef heart media and their influence on the growth of pneumococci; (2) the change in these initial carbohydrate contents and also in added glucose after the growth of pneumococci for different periods of time; (3) the change in the hydrogen ion concentration of beef heart broth after the growth of pneumococci; (4) the effect upon the growth of pneumococci of seeding into broths (with and without added glucose) of acidities corresponding to pH 6.0 to 7.7; will be presented in this paper.

EXPERIMENTAL METHODS

The broth used in these experiments was prepared as follows: 500 grams of chopped, fat-free beef heart were well mixed with 1 liter of water and allowed to stand overnight in the ice-box. The following morning the mixture was slowly heated to boiling and boiled for five to ten minutes until the meat was thoroughly clumped. The meat was then removed by straining through cheese-cloth. The volume of infusion was measured and 1 gram peptone (Fairchild) and 0.5 gram sodium chloride were added for each 100 cc. The peptone was disolved in a small quantity of cold water and added with stirring. Twenty cubic centimeters of normal sodium hydroxide solution per liter were added and the mixture brought to a boil. The additional required amount of normal sodium hydroxide solution to bring the mixture to pH 7.9 to 8.0 was added, as determined by preliminary tests using phenol red as indicator. The broth was then boiled for ten minutes, filtered through filter paper three times, bottled, and autoclaved for twenty minutes at 15 pounds pressure. The final pH was usually 7.4 to 7.7. Ten to fifteen liters of broth, made from three to five beef hearts, were prepared at a time. The beef hearts were obtained fresh from the slaughter house and chopped within an hour.

Estimations of the total nitrogen of the broth were made by the Kjeldahl method. For the carbohydrate or reducing action study, the protein of the broth and also of the broth fermented with washed yeast (Benedict (1928)), was precipitated by the Folin-Wu tungstic acid method (Folin and Wu (1919)). The reducing substances were determined in the filtrates from both the fermented and the unfermented broths by the Benedict sugar method (Benedict (1926)). In the Folin-Wu precipitation, because of the alkalinity of the broth and its low protein content, the usual proportions of acid and tungstate were slightly changed. The following amounts were found suitable in most cases: 8.0 cc. broth, 1.25 cc. 0.66 N sulfuric acid and 0.6 cc. 10 per cent sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) solution. Ten to fifteen drops of the tungstic acid filtrate were tested with two to three drops of a 0.4 per cent brom phenol blue solution in alcohol. The pH of the filtrate was usually 3.5 to 4.0. If it differed from this, the amount of sulfuric acid necessary to bring it to this pH was determined and another precipitation carried out with the corrected amount of acid. The recovery of glucose added to broth showed the method to be satisfactory for the present purpose.

The pH readings of the broths were obtained by testing them colorimetrically with a 0.02 per cent solution of phenol red in alcohol and a 0.04 per cent solution of brom thymol blue in alcohol as indicators (1 cc. of broth, 4 cc. of distilled water and 0.25 cc. of indicator solution). The densities of the cultures as indication of the ability of the various broths to support the growth of pneumococci were also studied. These densities were determined by comparison with the Kingsbury-Clark standards for albumin[1] which consist of a series of seven concentrations of albumin in tubes ranging from 10 mgm. (albumin) per 100 cc. equivalent to a very light growth of bacteria, to 100 mgm. (albumin) per 100 cc., which corresponds to a heavy growth. For example it was found that a density of about 20 mgm. with the test strain of type I pneumococcus which was used in these experiments corresponded to about 20 colonies in 0.000,000,1 cc. of the broth culture.

The virulence of the cultures was determined by mouse tests. The tests were carried out by intraperitoneal inoculation of the mice with 0.5 cc. of a culture diluted with broth mixed with 0.5 cc. of plain beef heart broth. As a rule four different dilutions of the standardized culture were used. These dilutions contained as nearly as possible the following number of living diplococci: 0.5 cc. of a 1:10,000,000 dilution of the broth culture containing 10 diplococci; 0.5 cc. of a 1:25,000,000 dilution containing 4 diplococci; 0.5 cc. of a 1:50,000,000 dilution containing 2 diplococci; 0.5 cc. of a 1:100,000,000 dilution containing 1 diplococcus. Each dilution was inoculated into 2 to 5 mice weighing between 16 and 22 grams. For the culture to be considered fully virulent the death in forty-eight to seventy-two hours of at least two-thirds of the mice inoculated with all four dilutions was necessary. Longer times of survival were considered to indicate diminished virulence. For an additional check, the protection of a standard antipneumococcus serum (Felton 146) against the cultures was tested.

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[1] Made by the Fales Chemical Company, Inc., Cornwall Landing, New York; K. G. Falk, G. McGuire, E. Valentine and E. Whitney, *Journal of Immunology*, 1931, 21, 199.

EXPERIMENTAL RESULTS

The total nitrogen contents (Kjeldahl method) of the beef heart infusions varied between 1.0 and 1.5 mgm. per cubic centimeter for the different preparations. The addition of the peptone brought the nitrogen in the finished broths up to 2.6 to 3.8 mgm. per cubic centimeter.

The reducing actions of the broths, estimated as described in the preceding section, were calculated either as milligrams glucose per cubic centimeter, or as per cent of glucose. Total and non-fermentable carbohydrates were determined, and the fermentable calculated by subtraction. Fermentable material appeared to be the most significant. It was, in all probability, glucose, and will be spoken of as such here, although it is possible that other fermentable reducing substances were present. The results refer to the growth of a strain of type I pneumococcus.

In table 1 are shown the results obtained in two series of experiments. The beef heart broths were made up as described and adjusted to different pH values. One set of broths was used directly, to the other set 0.5 mgm. of glucose per cubic centimeter (0.05 per cent) was added. Separate 10 cc. portions of broth were inoculated with 0.2 cc. and with 0.5 cc. of the supernatant culture of an eighteen-hour blood-broth culture (free from red blood cells) seeded from the heart blood of a mouse inoculated five to six hours previously. The densities, pH values, and total reducing actions calculated as milligrams glucose per cubic centimeter were determined after five and after twenty-four hours' incubation.

The initial reducing actions of the broths, calculated as milligrams glucose per cubic centimeter, were as follows:

	TOTAL	UNFERMENTABLE	FERMENTABLE
Broth 39 (February 18, 1931)	1.05	0.55	0.50
Broth 45 (March 18, 1931)	1.10	0.65	0.45
Glucose broth 39 (February 18, 1931)	1.58	0.55	1.03
Glucose broth 45 (March 18, 1931)	1.60	0.65	0.95

TABLE 1

Densities, reducing actions, and pH values of beef heart broths with different initial amounts of reducing actions and different pH values after pneumococcus type I growth

BROTH NUMBER	TIME OF GROWTH	INITIAL pH	RESULTS AFTER INDICATED TIMES OF GROWTH					
			0.2 cc. inoculum			0.5 cc. inoculum		
			pH	Density	Reducing actions (calculated as milligrams glucose per cubic centimeter)	pH	Density	Reducing actions (calculated as milligrams glucose per cubic centimeter)
	hours							
39	5	7.6	6.9	25		6.9	27	0.52
		7.0	6.8	15		6.7	25	0.50
		6.5	6.7	17		6.5	20	0.54
		6.0	6.2	Clear		6.0	15	
45	5	7.7	7.0	28	0.77	6.8	55	0.91
		7.0	6.9	22	1.07	6.7	25	0.99
		6.5	6.1	Clear	1.05	6.1	15	0.70
		6.0	5.9	Clear	0.71	6.0	Clear; 15	0.80
39	24	7.6	6.9	30	0.55	6.8	27	0.55
		7.0	6.6	27	0.53	6.7	25	0.52
		6.5	6.4	25	0.54	6.7	28	0.54
		6.0	6.2	Clear		6.0	22	0.53
45	24	7.7	7.0	35	0.65	6.9	35	0.62
		7.0	6.6	Clear; no growth; 30	0.62	6.7	15	0.66
		6.5	6.3	Clear (pn)	0.98	6.2	20; clear (pn)	0.71
		6.0	6.0	Clear; no growth	1.06	6.0	Clear (pn)	0.94
39 plus glucose	5	7.6	6.8	43	0.67	6.7	50	0.54
		7.0	6.8	Clear; 35		6.4	47	0.67
		6.5	6.7	Clear		6.3	35	0.66
		6.0	6.2	Clear		6.0	12	
45 plus glucose	5	7.7	7.0	30	1.02	6.7	55	1.21
		7.0	6.9	Clear; 50	1.38	6.5	38	1.47
		6.5	6.1	Clear	1.39	6.1	20	0.78
		6.0	5.9	Clear	0.80	6.0	Clear	1.13

Figures in the above table are the average of duplicate tubes. When the tubes did not agree closely, both results are given.

TABLE 1—*Concluded*

BROTH NUMBER	TIME OF GROWTH	INITIAL pH	RESULTS AFTER INDICATED TIMES OF GROWTH					
			0.2 cc. inoculum			0.5 cc. inoculum		
			pH	Density	Reducing actions (calculated as milligrams glucose per cubic centimeter)	pH	Density	Reducing actions (calculated as milligrams glucose per cubic centimeter)
	hours							
39 plus glucose	24	7.6	6.6	55	0.53	6.5	50	0.54
		7.0	6.8	Clear; 55	0.53	6.2	55	0.53
		6.5	5.8	Clear; 40	0.52	6.2	43	0.52
		6.0	6.2	Clear		5.3	44	0.53
45 plus glucose	24	7.7	6.7	Clear; 38 (2)	1.28; 0.64	6.7	38	0.62
		7.0	6.4	Clear; no growth; 12; 50	1.08; 0.62	6.5	Clear (pn); 40 (2)	0.63
		6.5	5.8	15; 12; no growth	0.91	6.1	25; 12; clear (pn)	0.69
		6.0	5.2	Clear; no growth; 15	1.37; 0.69	5.3	15 (pn); 15 (pn)	0.63

It will be seen that the results after five and twenty-four hours' growth were not always consistent. Part of such irregularity is due to the use of a series of tubes of broth seeded with inoculum from a common source and using a separate tube for each determination. Such a method is accompanied by experimental inaccuracies which may be additive and show unexpected and apparently unexplainable deviations in an otherwise orderly series.

Without going into the details of the experiments the following relations were indicated. The broths adjusted for optimum pneumococcus growth at pH 7.6 and 7.7 will first be taken up. The growths were invariably good here with somewhat heavier growths with the added glucose. With 0.5 cc. inoculum, the growth was heavier in a few cases than with the 0.2 cc. inoculum but the increase was not regular. Growth at the optimum pH was about the same in broths 39 and 45, as might be expected from the similarity in the reducing substance content. The fermentable reducing substances in broth 39 disappeared more quickly than those of broth 45, both with and without the added glucose, although the differences in the amounts of the reducing substances (fermentable and unfermentable) in the two broths were negligible. The decrease in the pH after fairly heavy pneumococcus growth was more marked in the broths to which glucose had been added than in the plain broths.

At the less favorable hydrogen ion concentrations for pneumococci, the growth was usually good at pH 7.0 (a few exceptions may be noted); not good as a rule at pH 6.5 with 0.2 cc. inoculum but better with 0.5 cc. inoculum; while at pH 6.0 occasional growth was observed.

Tests were also carried out using broth enriched with blood (0.2 cc. fresh citrated horse blood to 10 cc. of broth) inoculated with a loopful of heart's blood of a mouse infected with virulent pneumococci about five to six hours previously. Using the above blood broth procedure, with density determinations as the measure of growth, thirty lots of broth were tested for growth after eighteen hours.

These results showed that in addition to the initial pH of the broths, which had already been studied by others, the size of inoculum, the amount of fermentable carbohydrate in the meat infusion broth and the time the organisms were allowed to grow, are all interrelated factors which must be considered in determining the usefulness of a broth for culture purposes. Without going further into the details of the experimental conditions, the results obtained may be summarized as follows:

For satisfactory growth, the broth should have a fermentable carbohydrate content of 0.5 to 1.2 mgm. per cubic centimeter, calculated as glucose (or 0.05 to 0.12 per cent). For plain broth having this amount of carbohydrate and suitable pH, an inoculum of 0.2 cc. (supernatant blood broth culture, free from blood cells) per 10 cc. volume of broth gave satisfactory growth in five hours, the additional growth observed at twenty-four hours not being significant. In experiments not given in detail here, it was found that with blood broth (containing 0.2 cc. of citrated blood per 10 cc. broth) inoculated with a small loop full of heart blood of an inoculated mouse, growth was satisfactory with a somewhat smaller concentration of fermentable carbohydrate. With plain broth, satisfactory growth also occurred at times with smaller concentrations, but such growth was liable to be uncertain and at times scanty.

The most satisfactory initial pH was found to be 7.5 to 7.7. As stated, the amount of fermentable carbohydrate desirable was from 0.5 to 1.2 mgm. per cubic centimeter calculated as glucose. The formation of acid with accompanying cessation of growth was one of the controlling factors. For a virulent culture, if a short period of growth was desired, the carbohydrate content could approach the larger limit, if a long period, it should be at the smaller limit with phosphate added. The amount of fermentable carbohydrate was also determined at different time intervals, and it was found that usually practically all (within the experimental error) had disappeared in five to eight hours' growth, except perhaps with inordinately large amounts present originally. If growth continued after that time, it might have been due to undetermined minute amounts of fermentable carbohydrate or to other analogous substances present or formed in the broth. It must be remembered that the densities determined after eighteen or twenty-four hours, growth included both living and dead organisms. In addition a certain amount of autolysis of dead organisms might have taken place. It may be said therefore that a five to eight-hour culture of the pneumococci would in all probability represent a more active concentration of organisms than an eighteen-hour culture and should be more suitable for mouse protection tests in work with antibodies. On the other hand, for the formation of soluble specific substances, where a large amount of growth was desirable, considerably larger amounts of glucose and as much phosphate as possible might be used, since autolysis may be permitted, and, in fact, is desired here. In other words, where large numbers of organisms are required, rapid growth, with larger amounts of glucose, may be preferable.

The problem of the virulence of the cultures under the different conditions was more difficult to handle. The virulence was presumably maintained by growing the organism in suitable broth (blood broth, as a rule) for eighteen hours, then inoculating intraperitoneally into a mouse and at the end of five hours culturing the mouse heart blood in broth again for eighteen hours and repeating the cycle. This was done three or four times each week, the broth culture being kept at about 15° over each week end. The virulence was then tested with mice, as described in the preceding section.

In connection with the study of the virulence of the organism after growth in the various broths having different carbohydrate contents, the broths with small concentrations of fermentable carbohydrate (less than 0.5 mgm. per cubic centimeter calculated as glucose) gave irregular and often scanty growth. With blood broths (or green banana broths as shown in the following paper) containing 0.5 to 1.2 mgm. glucose per cubic centimeter virulence appeared to be maintained satisfactorily. At times the virulence appeared to be somewhat decreased, but it may well be that the eighteen-hour cultures were not as suitable for use for transplants as those of shorter growth periods. With larger quantities (1.6 mgm. of fermentable carbohydrate or more per cubic centimeter) growth was more luxuriant, but a tendency to grow in chains was noted. As stated earlier in this paper, the broths containing the larger amounts of fermentable carbohydrate tended to become acid more rapidly.

The virulence of the pneumococci is of importance especially in connection with their use in mouse protection tests, where it is desirable to have, as far as possible, optimum and reproducible conditions.

The preceding observations on the growth of virulent pneumococci in different preparations of broth show the importance of a preliminary testing of each broth, not only for optimum growth in it, but also for the total amount of reducing substance and the pH reached after eighteen hours' incubation if a culture of that age is desired. A broth having a high percentage of reducing substance (0.16 per cent or more calculated as glucose) is a source of danger since it appears satisfactory for good growth and the possibly harmful effects on virulence may be noticed only after several weeks. The danger line of pH concentration after eighteen hours' growth may be considered to be 6.5.

As a practical measure in the preparation of a beef heart broth as described, it was found that the estimation of the fermentable carbohydrate in the beef heart infusion would show whether the finished broth would be adequate in respect to fermentable reducing substance. The following results show this clearly:

	REDUCING ACTION (CALCULATED AS MILLIGRAMS GLUCOSE PER CUBIC CENTIMETER)		
	Total	Unfermentable	Fermentable
Infusion no. 20	0.53	0.45	0.08
Finished broth no. 20	0.70	0.60	0.10
Infusion no. 21	1.13	0.55	0.58
Finished broth no. 21	1.21	0.79	0.42
Infusion No. 23	1.15	0.50	0.65
Finished broth no. 23	1.29	0.65	0.64
Infusion no. 28	1.18	0.56	0.62
Finished broth no. 28	1.34	0.64	0.70
Infusion no. 30	0.71	0.42	0.29
Finished broth no. 30	0.84	0.60	0.24

For infusions low in fermentable reducing substance, the requisite amount of glucose may be added at the same time as the peptone before sterilization. In this way, a final broth may be obtained with the desired reducing substance content. Sterile glucose solution might be added to the final broth, if desired, or even solid glucose followed by an additional sterilization. However, it appears that the analysis of the meat infusion and addition of glucose to it if necessary, is the most satisfactory method.

CONCLUSIONS

The experimental results obtained with a strain of type I pneumococcus which are presented in this paper lead to the following conclusions:

1. The fermentable reducing substance content of beef heart broth gives a measure of the suitability of the broth for pneumococcus culture.

2. By setting the fermentable reducing substance calculated as glucose at 0.5 mgm. per cubic centimeter as the lower limit, the chances of a "poor broth" are greatly decreased.

3. Since the amount of fermentable reducing substance added in the peptone is negligible, the beef heart infusion may be analyzed for fermentable reducing substance; then, if necessary, the glucose may be added before sterilization to bring the total fermentable substance to the desired concentration.

4. *Under the conditions of pneumococcus growth used in these* experiments (10 cc. of broth with 0.2 or 0.5 cc. of culture) the fermentable reducing substance of the beef heart broths at pH 7.0 to 7.6 decreased rapidly most of it, usually, having disappeared at the end of five hours' incubation.

5. The unfermentable residue of the total reducing substance of the broth was practically the same after fifteen minutes fermentation by washed yeast and after eighteen to twenty-four hours' growth of pneumococci.

6. As the pneumococci grew and the fermentable reducing substances disappeared the broth became more acid. In general terms, the greater the amount of fermentable reducing substance the greater the production of acid as measured by the change in pH.

7. The lower pH limits at which pneumococci can be seeded and grow cannot be arbitrarily stated, but involve the correlation of the amount and condition of inoculum and the suitability of the broth, in addition to the hydrogen ion concentration.

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STUDIES ON ANTIBODIES

V. THE USE OF GREEN BANANAS IN MEDIA FOR THE CULTURE OF PNEUMOCOCCI

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The virulence of pneumococci *in vitro* is maintained, as a rule, by the use of blood broth (beef or beef-heart broth to which small amounts of sterile citrated horse blood are added) as a culture medium. It will be shown in this paper that green banana may be used in the medium in place of blood for this purpose.

EXPERIMENTAL

Beef heart broth was prepared as described in the preceding paper (McGuire, Valentine, Whitney and Falk (1932)).

Blood broth was prepared by the addition of 0.2 cc. of unhemolyzed, sterile citrated horse blood to 10 cc. of beef heart broth. The blood was not more than fourteen days old and was used preferably within one week after being obtained. Tubes of blood broth were freshly made once a week, incubated overnight at 37° to test for sterility, and then kept in the ice-box until needed. No tubes of blood broth older than seven days were used.

Green banana broth. Unripe bananas with green skins on which there was no yellow color were used.[1] The skin was rubbed with alcohol, air dried, and the skin cut away under sterile conditions (freshly boiled knife and forceps). Near the middle of the banana a circular cross-section as thin as practical, usually about 2 to 2.5 mm. in thickness, was cut. This cross-section was placed in a sterile petri dish and divided into 16 or 8 equal wedge-shaped pieces, (similar to the cutting of pie). The smaller wedge-shaped pieces having one-sixteenth the area of the circular cross-section weighed from 50 to 80 mgm. each. To make the "green banana broth," one of these wedge-shaped pieces was added to 10 cc. of beef heart broth. For most of this work size one-sixteenth was used, although one-eighth was equally satisfactory. The banana broth tubes were freshly made once a week, incubated over-night at 37°C. and then kept in the ice-box until used. No tubes more than one week old were used.

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[1] The green bananas were obtained through the courtesy of the United Fruit Company.

TABLE 1

Comparison of pneumococcus growth after eighteen hours in plain, green banana and blood broths

MEDIA (10 CC. PORTIONS)	INOCULUM					
	1 loop mouse heart blood			Supernatant blood broth, 0.2 cc.		
	pH	Densities	Reducing actions (calculated as milligrams of glucose per cubic centimeter)	pH	Densities	Reducing actions (calculated as milligrams of glucose per cubic centimeter)
Freshly prepared:						
Broth 28	6.7	47	0.48	6.7	42	0.48
Broth 28+ green banana	6.5	55	0.49	6.7	55	0.49
Broth 28+ blood	6.7	65	0.48	6.7	55	0.47
Broth 40	7.1	*	1.07	7.0	31	0.56
Broth 40+ green banana	6.9	49	0.56	6.9	50	0.57
Broth 40+ blood	6.9	65	0.56	6.9	60	0.55
After one week in ice-box:						
Broth 28	6.7	55	0.52	6.7	55	0.52
Broth 28+ green banana	6.5	70	0.49	6.5	55	0.50
Broth 28+ blood	6.6	75	0.49	6.5	60	0.49
Broth 40	7.4	*	1.05	7.0	47	0.55
Broth 40+ green banana	6.9	55	0.56	6.8	55	0.57
Broth 40+ blood	6.9	87	0.55	6.9	75	0.55

* Clear (no growth).

In table 1 are shown the results obtained in the eighteen-hour growth of type I pneumococcus in beef heart broth, green banana beef heart broth, and blood beef heart broth. Two different beef heart broths were used and tested when the banana and blood were freshly added and after standing for one week in the ice-box. Heart blood of a mouse inoculated five hours previously and the supernatant blood broth culture (free from blood cells) of a virulent pneumococcus were used for inoculation. The densities are given in terms of the Kingsbury-Clark albumin standards, and the reducing actions determined by the Benedict method as described in the preceding paper. The reducing actions are given in terms of glucose per cubic centimeter. Broth 28 contained reducing substances calculated as milligrams of glucose per cubic centimeter, total 1.34 mgm. unfermentable 0.64 mgm. and fermentable 0.70 mgm.; Broth 40, total 102 mgm., unfermentable 0.62 mgm., and fermentable 0.40 mgm.

TABLE 2

Comparative virulence tests of type I pneumococci grown in blood and green banana beef-heart broths

BROTH USED	NUMBER OF MICE TESTED					
	Dilutions of culture				Dilutions of control serum 146 mixed with 0.001 cc. of culture	
	0.0_75 cc.	0.0_72 cc.	0.0_71 cc.	0.0_85 cc.	0.0005 cc.	0.0002 cc.
Blood broth:						
Deaths <48 hours	11	9	13	9	0	12
Deaths 48 to 72 hours	6	8	4	2	1	3
Deaths 72 to 96 hours	2	1	0	1	2	0
Total deaths	19	18	17	12	3	15
Survivals	0	1	2	7	5	0
Green banana broth:						
Deaths <48 hours	13	8	11	12	3	12
Deaths 48 to 72 hours	9	10	8	8	4	2
Deaths 72 to 96 hours	3	4	1	1	1	1
Total deaths	25	22	20	21	8	15
Survivals	0	1	2	3	5	0

The green banana beef heart broths showed smaller densities as a rule than the blood beef heart broths. However, the differences were small and the growth sufficiently luxuriant in both the blood and the green banana beef heart broths. Growth in the plain beef heart broths was lighter and less regular. Where there was growth, the fermentable carbohydrate was used up in every instance. The acidity increase was not excessive in any case.

Since a satisfactory amount of growth was always obtained with the green banana broth, the question of the virulence of the culture was studied.

The virulence of the culture was tested with mice, as described in the preceding paper. The organism was grown in the broth in question for eighteen hours, then inoculated intraperitoneally into a mouse; at the end of five hours the mouse heart blood was cultured in the broth again for eighteen hours and the cycle repeated. This was done on three or four successive days each week, the broth culture being kept at about 15°C. over each week-end. After three weeks or more the virulence of the broth culture was determined by intraperitoneal inoculation of the mice with 0.5 cc. of the culture diluted with broth, mixed with 0.5 cc. of plain beef broth. The following dilutions were used:

0.5 cc. of a 1:10,000,000 dilution of the standardized broth culture containing about 10 diplococci
0.5 cc. of a 1:25,000,000 dilution
0.5 cc. of a 1:50,000,000 dilution
0.5 cc. of a 1:100,000,000 dilution

Each dilution was inoculated into from 2 to 5 mice weighing between 16 and 22 grams. As stated in the preceding paper, for the culture to be considered fully virulent, death of two-thirds of the mice within forty-eight to seventy-two hours was necessary. Longer times of survival indicated diminished virulence.

The results are summarized in table 2. Six separate series were run, two of these under the supervision of Miss Georgia Cooper at the Bureau of Laboratories of the New York City Health Department. As an additional check of the virulence of the cultures maintained on blood broth and on green banana broth, they were tested against a standard antipneumococcus serum (Felton 146). The colony counts are not given in detail in the table. They varied in the usual manner about the average values.

The results in table 2 show clearly that the virulence of the pneumococci was maintained fully as well on the green banana beef broth as on the blood beef broth. Data on culturing over a considerably longer period of time confirmed the fact of continued satisfactory virulence.

SUMMARY

The green banana as enriching material in place of fresh blood in beef heart broth for the culture of type I pneumococcus gave a good growth of organisms and in conjunction with mouse passage adequately maintained virulence.

REFERENCE

McGuire, G., Valentine, E., Whitney, E., and Falk, K. G. 1932 Journ. Bact. (Balt)., **23**, 223.

MILK-BORNE SEPTIC SORE THROAT AND SCARLET FEVER AND THEIR RELATION TO BETA HEMOLYTIC STREPTOCOCCI

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Since 1900, milk-borne epidemics of septic sore throat have been considered by the majority of physicians as comprising a clinical entity. It is upon this opinion of clinical entity that Davis laid his chief stress in his review of this subject given at the Portland American Medical Association meeting in the summer of 1929, when he reiterated his claim that the streptococcus isolated by him in 1912 from a milk-borne epidemic of sore throat and called by him *St. epidemicus*, is a distinct species and the cause of this disease.

From time to time, however, outbreaks have still been reported as giving a varying clinical picture; and scarlet fever as well as erysipelas and other septic conditions have been observed in the same epidemic.

Clear-cut records were not kept, in the earlier epidemics, of the incidence of sore throat and scarlet fever in districts surrounding the milk routes, and even in the affected district no record was made of the light cases of either, or of the number of cases who had had scarlet fever or severe sore throat previously. As early as 1902, Newsholme in his well-analyzed report calls attention to this omission as an unknown factor in the compilation of correct statistics of these conditions. Swithinbank and Newman in 1903 stated "in scarlet fever epidemics the ordinary sore throats are not reported and in sore throat epidemics the rash may be evanescent and desquamation scanty," and Smillie in 1917 states that in many septic sore throat epidemics scarlet fever cases also occur and that both types of cases may be traced either to cases of scarlet fever or to septic sore throat. Even as late as 1927, Armstrong and Parran emphasize the same indefiniteness in the epidemiologic reports; and among the best recent epidemiologic reports, such as Lombard's (1929), general statements are still made concerning the presence of "erysipeloid and scarlatiniform rashes" without further clinical details.

In the very striking outbreaks occurring in Massachusetts in 1928, and so well reported otherwise for the Lee epidemic by Lombard (1929), the following interesting—but still indefinite—statements on this point have been made respectively by Bigelow and White (1929) and Holmes (1929): "In children they were less severe and resembled those of first stages of tonsillitis and scarlet fever." "The clinical picture of the disease in children was unlike that in adults. The children were usually only mildly ill and seemed to have either follicular tonsillitis or scarlet fever. *St. epidemicus* was found in throat cultures taken from some of them." Nothing was said about these cases having had scarlet fever earlier or about the Dick test having been done.

The question of determining by the intradermal test the degree of susceptibility to the standard "scarlet toxin" and possibly to exotoxins made from other hemolytic streptococci and later to that of the epidemic strain may be of the greatest importance in helping to explain why we have so often the varied clinical picture reported.

We know that some scarlet fever strains produce more exotoxin than others and that some human beings react differently from others to different fractions of this toxin, and that when such a strain becomes implanted in a cow's teats we may have an outbreak classed as scarlet fever. We know that some strains of hemolytic streptococci produce more "endotoxin" than others, and that people react in varying degrees to this "endotoxin." We infer that epidemic septic sore throat strains are predominantly "endotoxin" producers, and that when such a strain is implanted into a cow's teats we may have an outbreak called septic sore throat.

The statement has often been made in connection with septic sore throat outbreaks that those individuals who had had scarlet fever developed only septic sore throat and, in connection with scarlet fever outbreaks, that those who had a history of erysipelas often developed no rash.

It is evident however, from a study of the published reports, that, even discounting a lack of clear-cut clinical detail, there must have been many large outbreaks of milk-borne disease where the majority, if not all of the victims had what might be called septic sore throat, and there must have been likewise a large number of outbreaks where the majority suffered from "scarlet fever," while a small but an appreciable number of outbreaks must have presented clearly both clinical pictures. The reports further indicate that very little work was done on the bacteriology of these outbreaks before 1885 when Klein found his streptococcus in a scarlet fever outbreak. After this work was discredited (erroneously in part) no work of any importance on the bacteriologic findings was reported until 1901. After that, streptococci were more or less regularly reported as present in "septic sore throat" outbreaks but no evidence was given that only one species causes these epidemics until the notable work of Davis and Rosenow in 1912. The bacteriologic evidence of Davis that his streptococcus is specific for the majority if not all epidemics of septic sore throat is based upon only two described differential characteristics. These are the appearance of a capsule in young moist cultures and the marked moisture and rapid drying of these cultures.

Many people since then have reported finding a streptococcus in septic sore throat outbreaks which, they said, was similar to *St. epidemicus* in all particulars, without describing those particulars. Some have even reported that investigators who, we find, never mentioned the word "epidemicus" in their article, had found *St. epidemicus*. Thus White (1929), in his recent fine review, states that Krumwiede and Valentine (1915) found a streptococcus of the epidemicus type (that overworked word "type") in the epidemic studied by them in 1915, in face of the fact that they were the second (Smith and Brown being the first), clearly to show that different epidemics of septic sore throat were due to different streptococci, as shown by the agglutination test. Smith and Brown compared their strains with some from the Chicago and Baltimore epidemics and showed that they were distinctly different in agglutination. They were alike only in being of the beta type of hemolysis which includes many groups classed as different species.

Smith and Brown and Krumwiede and Valentine are the only investigators up to 1929 who have reported a study of agglutination in connection with investigations on this subject and they used only direct agglutination. They clearly demonstrated that strains from different epidemics may have dissimilar agglutinogens.

These four workers were able to show by their work on agglutination that the probable first source of infection was a human case which infected the teats of the cow, assisted no doubt usually by some injury. They thus corroborated the work of Davis as to the cow being the direct source of infection, but went further in showing that it was probably never the original infection. None of them mentions capsules nor do they call any of their strains *St. epidemicus.*

The question then as to whether there is a species of hemolytic streptococcus that might be called *St. epidemicus* resolves itself again into the vexed question: What constitutes a species among bacteria? We all agree, in trying to answer this, that characteristics claimed as distinctive of a species must show a reasonably constant recognizable differential relationship to those of other members of the genus under similar conditions.

Some have claimed that the two characteristics upon which Davis bases his claim of specificity are sufficient. Thus Brown, Frost and Shaw (1926) state "this streptococcus (*St. epidemicus*) differs very strikingly and constantly from *St. pyogenes* in two major particulars, namely, *St. epidemicus* possesses a striking capsule when examined in moist India ink preparations and its colonies are large and moist and have a tendency to grow on the surface." This was reiterated by Frost et al. in 1929. Others say that these characteristics are so inconstant as to constitute only a variation (Smillie (1917), Walker (1923)). In fact, most investigators are in doubt whether to call a streptococcus showing these characteristics a species or a variety. Thus Brown and

Orcott (1920), say "the name *Streptococcus epidemicus* is used tentatively. The organism is so nearly like the *Streptococcus pyogenes* that it may be regarded as a well defined variety of the latter rather than a true species, and if so, it should be called *Streptococcus pyogenes* (variety *epidemicus*)."

Furthermore, some investigators have shown that strains from other sources, given other species names, may have similar characteristics (Jones and Little (1928), Wheeler (1930)). Even Brown, Frost and Shaw (1926) make this statement about a few strains classed as bovine and producing high acidity.

Then there are variations in the reports regarding the appearance and nature of the capsule. Some say there is no true capsule but only a "capsular substance" so variable as to be of little help in identification (Smillie (1917)). Even Davis and Rosenow (1912) said that their organism was usually encapsulated in body exudates but not in the throat.

Not only did Wheeler (1930, 1931) claim that strains from scarlet fever and erysipelas may show capsules, but she stated that strains classed as epidemicus may produce an exotoxin that can not be differentiated from "scarlet fever" exotoxin. We also have found this statement regarding exotoxin true. (See table 1).

We became interested anew in the subject of the relationship of strains of hemolytic streptococci to septic sore throat when Dr. Robinson kindly sent us three cultures from the Lee epidemic in August 1928. These were labelled: 4D from throat of patient; 76, from blood of patient who died; 27A, from cow suspected of having caused the epidemic. Three strains bearing the same numbers are listed in Robinson and Beckler's table, p. 228, as *St. epidemicus*. Beckler in November kindly sent us 14 strains from the Plymouth epidemic, 4 from the Charlton epidemic and 1 from Peter Bent Brigham Hospital labelled *St. epidemicus* (our number 309). Then Sobel brought from Boston, through the kindness of Dr. Robinson, 2 cultures,—one a strain from the 1911 epidemic in Boston (X32) and the other from the 1912 epidemic of Chicago (X40—Davis strain). These, with our own strains from related sources, make some 60 strains studied by us in this connection (see table 1).

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Showing relationship of hemolytic streptococ

DESCRIPTION OF CASE	SOURCE	NAME GIVEN BY ORIGINAL AUTHOR	I	
			2	308
308	Charlton epidemic, called septic sore throat	*St. epidemicus*	+	+
311	Charlton epidemic, called septic sore throat	*St. epidemicus*	+	+
320	Charlton epidemic, called septic sore throat	*St. epidemicus*	+	+
161	Sore throat from New York City	*St. hemolyticus*	+	
2	Type I strain from scarlet fever	*St. hemolyticus*, type I	+	+
245 (76)	Lee epidemic called septic sore throat; blood of dead patient	*St. epidemicus*	−	
246 (27A)	Lee epidemic; cow causing epidemic	*St. epidemicus*	−	
305	Plymouth epidemic called scarlet fever; patient	?	−	
306	Plymouth epidemic patient	?	−	
307	Plymouth epidemic patient	?		
310	Plymouth epidemic patient	?		
312	Plymouth epidemic patient	?		
313	Plymouth epidemic patient	?		
319	Plymouth epidemic patient	?		
323	Plymouth epidemic cow	?		
195	Milk-handler from Connecticut epidemic, 1926	?		
55	Subtype I strain from scarlet fever	*St. hemolyticus*, scarlet fever, subtype I	−	−
Leo	Aberrant scarlet	*St. hemolyticus*	−	
114	Scarlet fever type II	*St. hemolyticus*, type II	−	−
295	Epidemic of sore throat in New York Hospital for Contagious Diseases	*St. hemolyticus*	−	−
292	Epidemic of sore throat in New York Hospital for Contagious Diseases	*St. hemolyticus*		
293	Epidemic of sore throat in New York Hospital for Contagious Diseases	*St. hemolyticus*		
294	Epidemic of sore throat in New York Hospital for Contagious Diseases	*St. hemolyticus*		
176	New Jersey Hospital for Contagious Diseases, bad sore throat	*St. hemolyticus*		
256	New York State Dochez 5 (standard)	*St. hemolyticus*	−	
57	Type III strain from scarlet fever	*St. hemolyticus*, scarlet fever, type III	−	
Proveranz	Aberrant scarlet	*St. hemolyticus*		
28	Scarlet fever type IV	*St. hemolyticus*, scarlet fever, type IV	−	−
309	Peter Bent Brigham Hospital	*St. epidemicus*	−	
X32	Boston epidemic, 1911	*St. epidemicus*		
X40	Chicago epidemic, 1912	*St. epidemicus*	−	
Kent	Pneumonia blood	*St. hemolyticus*		
314	Plymouth epidemic	?	−	−
315	Plymouth epidemic	?		
316	Plymouth epidemic	?		
318	Plymouth epidemic	?		
322 (C27564)	Plymouth epidemic, cow	?		
108	Boston, 1917 epidemic of septic sore throat, cow	*St. epidemicus*		
244 (4D)	Lee epidemic throat	*St. epidemicus*		
White, G.	New York City Harlem Hospital, abscess of lung	*St. hemolyticus*		
Christopher	New York City Harlem Hospital, pneumonia	*St. hemolyticus*		
Bergland	New York City Harlem Hospital, pneumonia	*St. hemolyticus*		
267	Pneumonia, New York City	*St. hemolyticus*	−	
165	New York City, septic sore throat	*St. hemolyticus*		
Doyle	New York City Harlem Hospital, pneumonia	*St. hemolyticus*		
Milton	New York City, angina	*St. hemolyticus*		
B3	Erysipelas type II	*St. erysipelatis*, *erysipelas*, type II	−	
E9	Erysipelas type II	*St. erysipelatis*,	−	
C14	Erysipelas type II	*St. erysipelatis*	−	
C65	Erysipelas type II	*St. erysipelatis*	−	
C3	Erysipelas type I	*St. erysipelatis*, *erysipelas*, type I	−	
Medji	Bellevue Hospital, suspected sore throat	*St. hemolyticus*		
Greenberg	Babies Hospital doubtful scarlet	*St. hemolyticus*		
McKenzie	New York City, doubtful scarlet	*St. hemolyticus*		
163	Sore throat, New York City	*St. hemolyticus*	−	
170	Sore throat, New York City	*St. hemolyticus*		
297	Sore throat, New York City	*St. hemolyticus*	−	
Neal	Sore throat, New York City	*St. hemolyticus*		
321	Charlton epidemic	*St. epidemicus*	−	−

ABLE 1

from septic sore throat to those from other sources

AGGLUTININ ABSORPTION, OUR CLASSIFICATION

Scarlet types					Epidemicus types			Erysipelas types		Carbohydrate fermentation (Holman's types)*	Exotoxin		
Sub-type I	II	III		IV	I		II	I	II		Amount	Neutralization	Capsular space
55	114	57	295	28	309	X40	314	C3	B3				
+	−	−		−	−		−	−		*St. pyogenes*			
+	−	−		−	−		−			*St. pyogenes*			
+	−	−		−	−		−	−		*St. pyogenes*			
+	−	−		−				−	−	*St. pyogenes*	+	+	
+	−	−		−	−		−	−	−	*St. pyogenes*	+++	+	
+	−	−		−	−		−	−		*St. pyogenes*	+	+	
+	−	−		−	−		−	−		*St. pyogenes*	+	+	
+	−	−		−	−		−	−		*St. pyogenes*			
+		−			−		−	−	−	*St. pyogenes*			
+					−			−		*St. pyogenes*			
+		−			−					*St. pyogenes*			
+										*St. pyogenes*			
+										*St. pyogenes*			
+										*St. pyogenes*			
+	−				−		−	−	−	*St. pyogenes*	+++	+	+
+									−	*St. pyogenes*			
+	−	−	−	−	−	−	−	−	−	*St. pyogenes*	++++	+	+
−	+	−		−	−		−	−		*St. pyogenes*			
−	+	−		−	−		−	−	−	*St. pyogenes*	+	+	
−	−	+	+	−	−	−	−	−	−	*St. anginosus*	+	+	
−		+	+	−	−	−	−	−		*St. anginosus*	+	+	
−		+	+	−				−		*St. anginosus*			
−		+	+	−				−		*St. anginosus*			
−		+	+	−				−		*St. anginosus*			
−	−	+	+	−				−	−	*St. anginosus*	+++	+	
−	−	+	+	−			−	−	−	*St. anginosus*	+++	+	
−	−	−		+			−			*St. pyogenes*	−	−	
−	−	−		+	−		−	−	−	*St. pyogenes*			
−	−	−	−	−	+	+	−	−	−	*St. pyogenes*	+	+	+
−					+		−			*St. pyogenes*			
−					+	+	−			*St. pyogenes*			++
−	−	−	−	−	+		−	−		*St. pyogenes*			
−	−	−	−	−	−	−	+	−	−	*St. pyogenes*			
−		−	−				+	−		*St. pyogenes*			
−		−	−				+	−		*St. pyogenes*			
−		−	−				+	−		*St. pyogenes*			
−		−	−				+			*St. pyogenes*			
−		−	−			−	+	−	−	*St. pyogenes*			
−							+	−		*St. pyogenes*	+	+	
−	−						+	−		*St. pyogenes*			
−							+	−		*St. pyogenes*			
−							+	−	+	*St. pyogenes*			
−	−			−			+	−		*St. pyogenes*			
−							+	−		*St. pyogenes*			
−							+	−		*St. pyogenes*			
−	−						+	−	+	*St. subacidus*			
−	−	−		−			+	−	+	*St. subacidus*	+	+	++
−	−	−		−			+	−	+	*St. subacidus*			
−	−	−		−			+	−	+	*St. subacidus*			
−	−	−		−			+	−	+	*St. subacidus*			
−		−	−	−	−		−	+	−	*St. subacidus*	+	+	++
−		−	−	−	−		−			*St. pyogenes*			
−	−			−			−			*St. pyogenes*			
−		−		−						*St. pyogenes*			
−		−		−	−		−	−		*St. infrequens*			
−		−			−		−	−		*St. pyogenes*			
−		−			−		−	−		*St. pyogenes*			
−		−					−	−		*St. infrequens*			
−		−	−	−	−		−	−		*St. pyogenes*			

In the Charlton epidemic which was said by Scamman (1929) to be clinically septic sore throat, the source was believed to be a milk-handler, who alone cared for the cows (which were milked by machine but "stripped" by hand). He had had sore throat soon after the son's return from a hospital after recovery from scarlet fever. All four strains were tested with serum of scarlet fever type I as well as with the serum of our other type strains. The three culturally similar ones absorbed from the type I serum, the dissimilar one did not. Conversely, the type strain of scarlet group I absorbed from the serum from one of the strains (308), as did also another scarlet fever strain used to check the result,—one which cross-absorbed with scarlet fever type I.

Scamman (1929) stated that the Plymouth epidemic was undoubtedly one of scarlet fever, 101 cases being diagnosed as such, and that the probable source of infection was a milk-handler. Of the 14 strains received, eleven were from the throats of patients, one from that of the milk-handler, and two from cows.

The milk-handler strain and that from one of the cows were culturally alike and both, by absorption, belonged in subtype I from scarlet fever. The culture from the other cow was a mixture of a green-producing streptococcus with the hemolytic type. The latter was isolated by Bittman in our laboratory. This hemolytic constituent was found to belong to the agglutinating group of strain 314 (see table 1).

That a hemolytic strain from a cow absorbed from a scarlet group serum confirmed the finding of one laboratory of the two, to which, according to Scamman, the cow cultures and others were sent. This laboratory, Scamman states, reported that "one strain showed the characteristics, cultural and immunological, of the epidemicus type, while the cultures from the other cow showed the characteristics of the scarlet fever strain. The second laboratory reported the isolation of the epidemicus type from the 2 cows."

Scamman states that one of the two laboratories reported on the cow strains only, and he does not mention the other's report on the milk-handler.

Robinson and Beckler (1929) stated that the Lee (Massachusetts) epidemic of septic sore throat in June and July, 1928, was due to the milk of a cow with an infected udder; and that the source of the cow's infection remains unexplained although some evidence points to a milk-handler who had pneumonia about June 1. Shortly afterward, two of his children had sore throat. A culture from one of them, taken in July, gave a hemolytic streptococcus which did not resemble *St. epidemicus.* The organism from this cow's udder was (they state) *St. epidemicus.* The authors add that the strains are culturally identical with those from earlier epidemics—Boston, 1911 and 1917, and Chicago, 1912. Scamman makes the statement that "the same organism," identical in its cultural characteristics, was isolated from the throats of several patients and the blood of one.

All of the strains received through Robinson and Beckler were of the beta type of hemolysis except 1 that had some green-producing colonies. That several of the strains should not answer to the original statement that all colonies showed B. hemolysis emphasizes again the well known fact that in studying the relationship of strains, particularly epidemic or species strains, we come up against a number of conditions that may interfere with our demonstration of the chief strain at fault in any epidemic. Two of these sources of error, as we all know, stand out as of most importance: (1) the presence of a number of varieties of hemolytic streptococci already in throats upon which the epidemic strain may be implanted, the former often in large numbers since they may grow along with an implanted strain under the general conditions set up which are favorable for them. These may be isolated instead of the epidemic strain unless many colonies are fished and studied. (2) Any strain isolated may be in the process of dissociation and only a non-specific form may be isolated, or one containing only a fraction of the specific agglutinogens.

Of course, many other well known conditions may interfere with the securing of comparable results by different observers when working on the same epidemic or working later with presumably the same material.

CAPSULES OR CAPSULAR SPACES

We[1] have tested certain strains from septic sore throat, labelled originally *Streptococcus epidemicus,* and some of our agglutinative type strains from scarlet fever and erysipelas, in order to determine their comparative ability to produce a capsule or "capsular space" using a strain of type III pneumococci as a control.

We have tried four methods in our attempts to demonstrate capsules: (1) India ink moist and dried; (2) a new method just devised by Tyler of our laboratories; (3) Huntoon's method; (4) a combination of Huntoon's and Tyler's methods. After trying several culture media we found that the following gave the best results: Beef heart broth[2] + 0.5 per cent agar plus 8 per cent of whole horse blood (two days old) in citrate. Twenty-four-hour transplants from chocolate agar were used to streak surface plates of this medium, the growths being examined in eighteen hours. Macroscopically, the growth from all of these strains resembled that described for *Streptococcus epidemicus*—a moist irregular growth along the streak.

With the India ink method, carried out according to the directions of Brown and Orcutt, all of the streptococci which we tried gave a clear-cut capsular space, none of them equalling the pneumococcus type III; but none of the strains marked epidemicus showed up better than our type strains from scarlet fever and erysipelas.

We tried Huntoon's method because Brown and Orcutt reported that they had been able to demonstrate a capsule by this method, but we obtained no clear-cut pictures. We also tried a method Tyler had just begun to use for his students. This method, too, gave indefinite pictures. Then Miss Mann happened to use the nutrose of Huntoon's method with Tyler's method, with the result that the capsular space and often a capsular line stood out clearly.

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[1] A. G. Mann, one of our bacteriologists, had made the required cultures and stained the specimens for us with the results recorded in the text.

[2] Park, Williams and Krumwiede; "Pathogenic Microörganisms," 9th edition 1929, page 98 for preparation of beef heart broth.

The staining method is as follows:

Crystal violet	0.1	gram
Acetic acid glacial	0.25	cc.
Water to make	100	cc.

Three per cent nutrose solution in distilled water. Cook in Arnold for one hour (do not filter) and add 0.5 per cent carbolic. Mix organisms in a drop of nutrose solution, air dry, do not fix. Cover with stain, allow to stand for four minutes, wash quickly with 20 per cent copper sulphate (aqueous solution), dry on filter paper.

Probably this method would help to demonstrate a "capsular space" surrounding many bacteria grown under similar conditions.

It was clearly shown that the type strain of one erysipelas group (strain C3) possesses a capsule when in the animal body. Thus spreads from the peritoneum of the forty-eighth passage-mouse showed (Wright's stain) distinct capsules. That we were dealing with the streptococcus injected and not with some other hemolytic streptococcus which might possibly have been present in a seemingly normal mouse, was evidenced by the isolation of the organism in pure culture and its power of absorbing completely the agglutinins from the serum of erysipelas type I. The culture from passage-mouse 47, with which mouse 48 was injected, had been kept on artificial media for many months.

The fact that capsular spaces and even capsules may be produced so easily and so non-characteristically by us with streptococci that have been definitely classed, some with the scarlet fever group and others with the erysipelas group, coupled with the fact that streptococci from septic sore throat that have been shown by us and others to belong to our largest scarlet fever group by agglutinin absorption, have been labelled by their discoverers *St. epidemicus* because they have shown a "capsular space" in moist India ink preparations when first isolated, constitutes evidence that discredits the demonstration of a capsular space as an indication of species within the group of hemolytic streptococci.

AGGLUTININ ABSORPTION

The following is a brief account of the technic we found best for our absorption tests.

Stock cultures. These have been grown on vitamine heated blood agar ("chocolate" agar), containing 5 per cent citrated horse blood, and stored in the ice-box; one sealed tube of each strain was, however, kept at room temperature. Unsealed ice-box tubes were transplanted once in seven or eight weeks; sealed tubes of almost all strains may remain untransplanted for a much longer time. The mouse-passage stock was grown on veal agar slants on which a drop of horse blood had been placed.

Sera. For a dependable test, irreproachable serum and antigen are necessary. In these studies rabbit sera only were used. Generally speaking a high titer serum (around 3000) has given the most clear-cut results. Such a serum has been obtained from the majority of our rabbits and most of our strains. Healthy, young (2000 to 2500 grams) male rabbits with good ear veins are chosen. These are injected intravenously with live cultures. The first injection is usually 0.1 cc. of a twenty-four-hour growth on vitamin-agar slant in potato tube suspended in 2 cc. 0.85 per cent salt solution. The injections are given daily for four days, with a rest of three days. The increase in the amounts injected as well as in the number of injections are regulated according to the weight and general condition of the animal. A satisfactory serum is usually obtained in from four to six weeks.

Preparation of antigen suspension for agglutination tests. The smallest number of generations which will yield a suspension that is not self-agglutinating is to be preferred. The medium used is 0.2 per cent phosphate broth containing 0.1 per cent glucose to enhance growth. To hasten cessation of self-agglutination we transplant (by pipette) each morning and noon. If the strain is highly self-agglutinating we remove the generation planted at noon to the ice-box before leaving (Rachstein's suggestion) or transplant a third time. The usual procedure is to plant a culture from this generation in 0.2 per cent phosphate broth poured over autoclaved strips of potato and to incubate this five to six hours. The cells are then collected by centrifuging, washed in 0.2 per cent phosphate broth to remove possible deleterious products and, after packing and removal of washing fluid, emulsified in 0.5 per cent phosphate broth. For convenience, the emulsion may be kept in the ice-box over night. In the morning the upper portion is drawn off from any sediment and, if visible particles are present, is very briefly centrifuged to throw these down. More 0.5 per cent phosphate broth is added if greater dilution is desired.

Stevens cautioned against the use of new potatoes and we add that the potato must not be too long in the broth, as specificity of flocculation is interfered with. The broth is poured over the potato at the time of planting.

Suspensions made by growing the last generation as well as previous ones in the phosphate broth with 0.1 per cent glucose, at times work better than when the cells are grown in potato broth. In this case the culture is planted the last thing before leaving, the cells are collected the first thing in the morning, and are washed and suspended as described for potato-broth cultures.

Performance of test and reading of results. The tube method has been used because it seems to be more delicate than that of hanging drops. The use of a much thinner antigen suspension, acted upon by the serum for a longer time, gives agglutination over a wider range of serum dilutions, and the higher temperature gained by the water bath, probably hastens the reaction.

For diagnostic purposes, early judgment may be had by examination of the contents of the tubes in hanging drops. Greater delicacy in judging the degree of absorption, if any, may be had by observing the tubes after settling of the clumps, using a hand lens with which to compare the size and number of clumps caused by the different dilutions of serum.

In the earlier period of the work sera to be absorbed were always diluted 1:10. Phosphate broth has always been used for diluting (0.2 per cent). Later in the work, sera of high titer were, in a number of cases, used in higher dilution (1:20, occasionally 1:30) since it seems better not to have a very large proportion of cell-volume to the volume of the diluted serum, and without that there was risk of incomplete absorption by the homologous strain.

The minimal absorbing dose of each serum was determined, and two or two and one-half times that dose was used in the test, following Krumwiede's recommendation.

Absorption was done at 45°C. for two hours, with almost constant agitation of the cells for the first ten minutes, and at intervals thereafter.

In setting up the racks after an absorption, 8 dilutions of serum were used, representing, in the majority of cases, amounts of straight serum from $\frac{1}{100}$ to $\frac{1}{12,800}$ cc. In the later work, the first tube represented a 1:50 dilution. Dilutions as low as 1:10 have been used when necessary.

Time of reading absorption tests. Observations of the tubes during first one to three hours is undoubtedly desirable as important differences among strains is evident, in size and rate of clumping by the same serum after absorption by them. If the serum is good in titer and reacts promptly with the antigen, that is, if the antigen suspension is also of good character, the afternoon reading suffices. If either condition is not up to standard, the morning reading is necessary. Occasionally a discrepancy is observed between the two, in which case the test is repeated until satisfactory results are obtained.

We may see from a study of table 1 that by agglutinin absorption, we have obtained some very interesting results.

The four strains from the Charlton epidemic are of particular interest because three of them appear to have fallen into our type I scarlet fever group, a comparatively small group. But all of these strains have given evidence of being in a state of dissociation, showing coarse and fine (rough and smooth colonies) on blood agar plates; therefore clear-cut absorptions with the cultures as originally received by us could not be expected. We are still working on the relationship of these strains. One (321) was rather clearly different from the other 3 culturally and proved to be so serologically.

All of the strains classed as anginosus by Holman's carbohydrate classification fell into our scarlet fever type III agglutinative group. Four of these came from an outbreak of sore throat in a scarlet fever hospital (to be reported).

Of the other strains (most of which are classed as pyogenes by Holman's classification) ten fall into our scarlet fever sub-type I (forming our largest group from scarlet fever). Of these, 2 came from the Lee epidemic called septic sore throat and 8 came from the Plymouth epidemic of scarlet fever. Two of these complete absorbers were from cows, 1 from the Lee epidemic and one from the Plymouth epidemic. This corroborates the demonstration by Jones and Little (1928) that a scarlet fever type of hemolytic streptococcus may become implanted into cows udders—a fact which we also had shown independently.

The type strain of what we have called epidemicus type I (strain 309) came from a case diagnosed at Peter Bent Brigham Hospital, Boston, as acute follicular tonsilitis with peritonsillar abscess. This was in October, 1929. When lanced, the abscess yielded 20 cc. of pus. The patient, twenty-four years old, gave no history of previous scarlet fever. The second of the 4 cases whose strains absorbed from the serum of this strain was from the Boston epidemic of 1911 (X32), the third from the Chicago epidemic of 1912 (Davis X40)—showing the widespread distribution of this type. We have prepared a serum from the Davis strain and have used it in making cross agglutininations and absorptions in this group. The fourth case in this group was from a terminal blood infection after pneumonia. On our regular blood-agar streak plates, the type strain clearly produces colonies that are commonly more moist than those from scarlet fever strains used.

The four strains from Plymouth which did not absorb from scarlet sub-type I, and were unlike this type culturally, were found all to absorb from a serum of one of these (314). We have called this epidemicus type II.

Other strains belonging in this group are: The strain from a cow, designated by the Massachusetts Department of Health, Cow 108, which was considered to be the source of an epidemic of septic sore throat in Boston in 1917, reported upon by Brown and Orcutt (1920) who designated the strain *St. epidemicus*.

Strain 165 from the throat of an adult (Dr. L. of Hebrew Infant Asylum, New York City) who had a sore throat with fever in 1927. Dr. L. gave a history of scarlet fever in childhood.

Strain 244 (4D) from the Lee epidemic.

Five strains each respectively from 5 cases of pneumonia (to be reported).

One of the most interesting facts about this group, indicated in the table, is that the strains of erysipelas type II absorb the agglutinins from the serum of this type; while the strains from this epidemicus type II group do not absorb the agglutinins from the serum of erysipelas type II; that is, the epidemicus type II group is a sub-group of erysipelas type II group. As in the case with strains belonging to the (apparently smaller) absorption group represented by strain 309, we notice the wide distribution of these types.

These results, added to the epidemiological studies, illustrate forcibly what we may expect when the ubiquitous hemolytic streptococcus comes in contact with milk and when neither the milk nor the human nor cow carriers are properly controlled—one to several varieties of streptococci may be present and active in any epidemic.

Practically the question of calling a strain of hemolytic streptococci isolated from milk or elsewhere "*St. epidemicus*" is unimportant, except as it may be harmful in making us forget that there are other strains that may not show the characteristics ascribed to them, and that do cause a septic sore throat outbreak (Charlton), and that there are strains with similar characteristics that may be non-virulent. It is true that it may help us to find an epidemic strain, but it may also help us to lose one if there are other varieties present. If we want to find the epidemic strain we must determine the absorption of agglutinins, which is still the most effective method used for demonstrating an epidemic strain.

At this stage of our knowledge concerning the discovery and control of carriers (either humans or bovine), of pathogenic streptococci, we must say, with others, that the only effective practical measure for controlling milk-borne streptococcic diseases, as well as other milk-borne diseases, is efficient pasteurization.

SUMMARY

1. A short review of the present status of "*St. epidemicus*" as a species is given and evidence is presented to show that neither of the characteristics claimed as the only species characteristics—capsule formation and moist growth on blood media—holds sufficiently to be used in designating a species and that at present strains of hemolytic streptococcus from septic sore throat not otherwise placed by agglutinin absorption should be termed simply *St. hemolyticus*.
2. Strains of hemolytic streptococci from milk-borne epidemics of scarlet fever and of septic sore throat, and from other sources have been studied to determine their serologic relationship to our type strains from scarlet fever and erysipelas and to each other.
3. The strains from septic sore throat in the three Massachusetts outbreaks of 1928 were shown to fall by agglutinin absorp-

tion partly into our largest scarlet fever group type I and subtype I and partly into two other groups. These two newly discovered agglutinative groups we have called epidemicus (or septic sore throat) type I and epidemicus (or septic sore throat) type II.

4. Septic sore throat strains and scarlet fever strains may occur in the same epidemic, composed of cases classed mostly as scarlet fever.

5. A scarlet fever type of organism may be recovered from cases recorded clinically as constituting a septic sore throat outbreak.

6. Exotoxins of similar quality as regards their neutralization ability, but of varying quantity have been produced by all of these type strains.

CONCLUSION

We conclude from our studies on this subject that milk-borne epidemics of septic sore throat and of scarlet fever may be caused respectively by one or several agglutinative types of hemolytic streptococci. When scarlet fever predominates, usually the epidemic strain belongs to one of the common scarlet fever agglutinative types; when septic sore throat predominates, the epidemic strain may be one of several agglutinative types respectively, two of which have been demonstrated in these studies and have been called tentatively epidemicus agglutinative type I and epidemicus agglutinative type II.

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THE BIOCHEMICAL CHARACTERS OF HUMAN AND ANIMAL STRAINS OF HEMOLYTIC STREPTOCOCCI[1]

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There have been numerous efforts to discover a biochemical method of differentiating streptococci of human and animal origin. In part, these have been successful. The work of Ayers (1916), Ayers, Johnson and Davis (1918), and Avery and Cullen (1919) has demonstrated that certain hemolytic streptococci of bovine origin can be differentiated from human streptococci by the final hydrogen-ion concentration produced when they are grown in glucose broth. Working with the same group of bovine streptococci, Ayers and Rupp (1922) were able to separate them from streptococci of human origin by their ability to hydrolyze sodium hippurate. Avery (1929) also demonstrated that the members of this particular group of bovine streptococci were tolerant to concentrations of methylene blue which inhibited streptococci of human origin. Frost and his associates (1926, 1927, 1929) have carried out extensive studies of streptococci of bovine origin and classified a large number of strains.

There remains however a large group of hemolytic streptococci derived from bovines and other domestic animals which has not been successfully differentiated from human streptococci. This group of streptococci is characterized by low acid production in glucose broth, inability to hydrolyze sodium hippurate, lack of tolerance to methylene blue, and active hemolysis of blood cells in a fluid medium. These characters are likewise possessed by streptococci of human origin. It is with this group of undifferentiated animal strains that the present paper is concerned.

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[1] The investigation reported in this paper was carried out in connection with a project of the Kentucky Agricultural Experiment Station, and is published by the permission of the director.

The cultures studied consisted of a group of 90 strains of streptococci isolated from horses, cattle, swine and chickens, together with 36 strains of human origin used for comparison. These cultures all produced a relatively low acidity in glucose broth, the final hydrogen ion concentration never exceeding pH 4.9.

TABLE 1

Source of cultures

Animal strains:	
Equine, os uteri	54
Equine, aborted fetuses	3
Equine, septicemia and arthritis	11
Equine, pneumonia	2
Bovine, septicemia	3
Bovine, placenta	1
Bovine, milk	1
Chicken, femur	9
Swine, septicemia	2
Swine, abortion	4
Human strains:	
Throat cultures, sore throat and scarlet fever suspects	19
Broncho-pneumonia	4
Scarlet fever, stock cultures	7
Mastoiditis	3
Meningitis	1
Erysipelas	1
Smallpox	1

In contrast to this, the so-called high acid producing strains have, in our hands, produced acidity ranging from pH 4.4 to 4..0 None of the cultures hydrolyzed sodium hippurate and all were markedly hemolytic in a fluid medium. The question of hemolysis deserves some consideration. While high acid-producing, sodium hippurate-hydrolyzing streptococci may be hemolytic to a certain degree and produce fairly well-defined zones of clearing on a blood plate, they are not, in our experience, nearly so markedly hemolytic as the group under consideration. The hemolytic activity of all the strains studied here has been roughly titrated. As a result it has been found that 0.05 cc. of the supernatant fluid of centrifuged broth cultures of these streptococci is able completely to hemolyze 0.2 cc. of a 5 per cent suspension of washed sheep cells. The high acid-producing cultures of bovine origin have not proved nearly so active against red cells, the majority of them not producing complete hemolysis in amounts of 0.5 cc. The source of the strains studied is given in table 1.

ACID PRODUCTION

While it has been stated above that all the cultures reported upon were of the low acid-producing type, different degrees of acid production from glucose have been noted in the human and animal strains. In determining the final hydrogen ion concentration of the cultures, meat infusion broth, adjusted to pH 7.8 and containing 1 per cent glucose, was inoculated with the strains to be tested and incubated at 37°C. for ninety-six hours. The hydrogen ion concentration was then determined by comparison with standard buffer solutions. It was found that the average hydrogen ion concentration reached by the animal strains was pH 5.1 while that attained by the human strains was only pH 5.84. The reaction of the individual strains is given in table 2. With the exception of seven cultures the difference observed between the human and animal strains is distinct and consistent and seems to indicate a difference in the metabolism of the cultures. It is of interest in this connection that Jones (1919) noted that strains of hemolytic streptococci of equine origin

TABLE 2

Final hydrogen ion concentration in glucose meat infusion broth cultures of streptococci

	pH											
	4.9	5.0	5.1	5.2	5.3	5.4	5.5	5.6	5.7	5.8	5.9	6.0
Animal strains..........	3*	33	12	42								
Human strains..........						3		4		10	4	15

* Figures indicate number of strains at various hydrogen ion concentrations.

produced a higher titrable acidity in carbohydrate mediums than did human streptococci.

FERMENTATIVE REACTIONS

The fermentative reactions of streptococci have been studied extensively and in general the results have been discouraging. This method of study, once regarded as being of great promise, has proved disappointing. There are a few notable exceptions, however, such as the studies of Holth (quoted by Adsersen (1915)), Adsersen (1915) and Ogura (1929) on the fermentative characters of *Str. equi*. These workers have examined more than 250 cultures of this species and found their action on fermentable substances absolutely constant. Not only did all strains of the microörganism exhibit the same fermentative reactions but these reactions differed from those of human streptococci and of other strains of equine streptococci. These observers found that *Str. equi* failed to ferment lactose, sorbitol and trehalose. All the other cultures examined fermented one or more of these substances. In addition Ogura demonstrated that the majority of equine strains fermented sorbitol and failed to ferment trehalose, while the few human strains which he examined fermented trehalose and failed to ferment sorbitol. Since these results offered promise of a method of differentiation, Ogura's studies have been extended to a larger number of strains from a greater variety of animal species.

In the present investigation the 90 strains of animal origin and the 36 human strains have been tested for their ability to ferment sucrose, salicin, lactose, sorbitol, arabinose, xylose, sorbose, trehalose, mannitol, raffinose, rhamnose, dulcitol, glycerol, and inulin. In performing the tests the following basic medium was used:

Meat infusion	900 cc.
Casein digest	100 cc.
Andradés indicator	1 per cent

The casein digest was prepared according to the method of Kulp and Rettger (1924). The reaction was adjusted to pH 7.6 and the medium sterilized. The fermentable substances were sterilized by filtration and added to the previously sterilized broth. After inoculation the tubes were incubated ten days before final recording of results.

Of all the substances tested, only lactose, trehalose, sorbitol and glycerol proved to be of any value for differentiation. The results obtained with these substances are given in table 3. It can be seen that the 36 human strains without exception formed acid from trehalose but did not attack sorbitol. On the contrary, 85, or 94 per cent, of the animal strains failed to ferment trehalose but produced acid from sorbitol. The remaining five animal strains reacted as did the human strains in regard to these two substances. It will be noted, however, that the percentage of lactose fermenters in this group is much lower than in the human cultures. Only one of these five animal strains fermented lactose, while 34 of the 36 human cultures produced acid from this sugar. Also the percentage of glycerol fermenters is higher, 100 per cent of the atypical animal strains attacking glycerol, whereas acid was formed from this substance by only 50 per cent of the human cultures. It is of interest that no streptococcus has been encountered which ferments both trehalose and sorbitol. With the exception of *Str. equi*, which is not considered in this paper, we have not found a strain which fails to ferment one of these two substances, one or the other always being attacked. We have confirmed the statements of Holth, Adsersen and Ogura that *Str. equi* ferments neither lactose, sorbitol nor trehalose.

TABLE 3

Fermentation reactions

		LACTOSE	SORBITOL	TREHALOSE	GLYCEROL
Animal strains	85	+	+	−	−
	5	1+, 4−	−	+	+
Human strains	36	34+, 2−	−	+	18+, 18−

+ indicates acid production; − indicates no acid production.

DISCUSSION

It has been shown above that the animal strains examined in this work produced a higher acidity in glucose broth than did the human strains. However, this method may not be of practical value since some of the human strains produce almost as great a degree of acidity as do the animal strains. The final hydrogen ion concentration produced by the 36 human strains included in this group is lower than that reported by many workers for human streptococci. Numerous references may be found in the literature to human streptococci which produce a final reaction of pH 5.0 to 5.2 in glucose broth. We are unable to explain the lower acidity produced by our series of human cultures. The work has been repeated several times and the results confirmed by other persons in the laboratory. The rather constant differences observed are indicative of a basic difference in the metabolism of the organisms.

In the great majority of instances the results of the fermentation tests were sharp and distinct. The only doubtful or delayed fermentations obtained were in the tests with glycerol. In testing lactose, trehalose, and sorbitol positive reactions were nearly always apparent in twenty-four hours and were never delayed beyond forty-eight hours. Glycerol was fermented very slowly in these tests and in some instances the production of acid was very slight. Positive tests with this sugar were rarely apparent before the fourth or fifth day. It should be stated that the most vigorous glycerol fermenters in this series were the five atypical animal strains. They produced acid earlier and in greater amount than any of the human cultures.

The question naturally arises as to whether the five atypical strains of animal origin may be in reality human strains. It is the writer's opinion that they are not. Three of these atypical strains were recovered from the *os uteri* of mares, one from a case of septicemia in a foal, and the fifth from an aborted swine fetus. These five cultures grow much more vigorously in artificial media than the other animal strains and somewhat more vigorously than the human cultures. They produce higher acidity in glucose broth than the human strains. As pointed out above the percentage of lactose fermenters is much lower and the percentage of glycerol fermenters higher in these five cultures than in the human strains. These facts indicate that they are not human strains of streptococci which have been transferred to animals. If the results obtained in this study can be confirmed with a larger number of cultures it may be said with certainty that any hemolytic streptococcus producing acid from sorbitol and failing to ferment trehalose is of animal origin. On the other hand if a strain produces acid from trehalose and fails to ferment sorbitol, it is highly probable that the organism is of human origin.

The study of this series of cultures is being continued in the hope that additional differences may be established between the human and animal cultures and an exact method found of differentiating the atypical animal strains from the human type.

In conclusion it should be said that the results obtained here apply only to the group under consideration and the method is not applicable to other groups of streptococci. The few high acid-producing, sodium hippurate hydrolyzing strains which have been tested do not give concordant results.

SUMMARY

In a series of 126 strains of hemolytic streptococci, 90 of animal and 36 of human origin, it has been demonstrated that the animal cultures produce a higher acidity in glucose broth than do the human cultures. It has also been demonstrated that 94 per cent of the animal strains can be clearly differentiated from the human cultures by the fact that they produce acid from sorbitol and fail to ferment trehalose. The streptococci of human origin all ferment trehalose and fail to attack sorbitol.

The writer wishes to take this opportunity to express his grateful appreciation to Dr. W. W. Dimock and Dr. D. J. Healy for their interest, coöperation and helpful suggestions throughout this work.

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ATYPICAL (SLOW) LACTOSE FERMENTING B. COLI

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During studies on infectious diarrhea (Jones and Little (1931)) in cows, motile Gram-negative rods which failed to attack lactose were frequently encountered among the colonies developing on the surfaces of lactose agar plate cultures streaked with fecal suspension. Their numbers varied greatly; at times they made up 90 per cent of the organisms, at others they were present in relatively small numbers and in many cases no such colonies were found. In one instance they were found throughout the small intestine of a cow slaughtered during an attack of diarrhea. At first it seemed to us that we had to deal with organisms of the paratyphoid group but sharp differences in agglutination affinities between these organisms and both types of animal paratyphoid indicated little immunological relationship. In addition, cultural differences were readily manifested.

Many have reported the presence of atypical non-lactose-fermenting *B. coli* in the intestinal and urinary tract of man and in a few instances they have been noted in the feces of horses and cattle. Gilbert and Lion (1893), appear to have been the first to describe Paracolon bacilli. Morgan and Ledingham (1909), encountered such types in human feces and divided them into three groups. On the basis of cultural characters they reported them as numerous in cases of diarrhea but regarded them as of little pathogenic significance. On the other hand, György (1920) suggested that they played a considerable part in diarrhea in both man and calves. Stewart (1926), who studied the Mendelian variation in organisms of this group, recognized that two distinct types existed: (1) paracolon bacilli, which fail to attack lactose after long periods, and (2) mutable colon bacilli which possess a routine Mendelian variation with respect to the fermentation of such substances as lactose, sucrose and dulcitol. In this respect Stewart broadened the original definition of *B. coli-mutable* which Neisser (1906) and Massini (1907) applied to the organisms whose action on lactose only was variable.

THE SOURCE OF ATYPICAL COLON BACILLI

In our earlier studies on infectious diarrhea of cows we encountered in the feces actively motile organisms which, on the surface of lactose agar plates, failed to attack the carbohydrate. In certain outbreaks all specimens of feces contained them while in others their appearance was irregular and in later epidemics during 1930 and 1931 we failed to find them. In addition to our own isolations, Dr. W. A. Hagan (1925), Professor of Bacteriology at the New York State Veterinary College, Cornell University, supplied us with several strains which he had obtained from intestinal material, pond water, etc., during his study of Braken poisoning of cattle. Furthermore, we examined the feces from 50 normal cows drawn from 5 herds in this vicinity and cultivated atypical colon bacilli once. Another culture was isolated from the urinary tract of a healthy calf. From this it would appear that such organisms are not present in large numbers in the intestinal tract of normal cows although they may be the predominating types during certain intestinal disorders; but, as we have shown, there is little evidence to incriminate them as the etiologic agent in such maladies.

THE CULTURAL CHARACTERS OF THE GROUP

When the surfaces of agar plates, containing brom-cresol-purple, in addition to 1 per cent lactose, are streaked with feces or culture, smooth round translucent colonies of actively motile bacilli develop. The surrounding medium retains its purple color even after incubation or storage in the room for two weeks or more. In our hands, buds or protrusions which ferment lactose have not occurred, although strains may, as will be shown later, under certain conditions, develop both rapid lactose and slow lactose fermenters. When digest broth containing carbohydrate and indicator (brom-cresol-purple) is inoculated with culture, many of the carbohydrates (table 1) are promptly fermented with acid and gas production. Lactose broth, however, remains alkaline for varying periods. When the cultures were freshly isolated, there was no appreciable change in reaction during the first three or four days of incubation and, in some cases, during the first two or three weeks. One strain isolated from the urine failed to attack lactose, as judged by acid production and gas formation, during thirty days. However, after repeated transfer it now begins to ferment this substance after four or five days. As is brought out in table 1, all strains readily attacked glucose, mannitol, maltose, and xylose, with the production of acid and gas, and failed to ferment raffinose, but fermented lactose slowly. In addition all gave a negative Voges-Proskauer reaction, produced indol, gave a positive methyl-red reaction, and failed to liquify gelatin or produce H_2S. In the fermentation of such substances as sucrose, dulcitol, and salicin, differences were noted as indicated in table 1.

TABLE 1

The biochemical characters of atypical colon bacilli

NUMBER OF STRAINS	GLUCOSE	LACTOSE	SUCROSE	MALTOSE	MANNITOL	XYLOSE	RAFFINOSE	SALICIN	DULCITOL	VOGES-PROSKAUER	INDOL	METHYL RED	LIQUEFACTION OF GELATIN	PRODUCTION OF H_2S
7	A.G.	Slow fermentation	A.G.	A.G.	A.G.	A.G.	0	A.G.	A.G.	–	+	+	–	–
1	A.G.	Slow fermentation	A	A.G.	A.G.	A.G.	0	A.G.	A.G.	–	+	+	–	–
1	A.G.	Slow fermentation	A.G.	A.G.	A.G.	A.G.	0	A.G.	0	–	+	+	–	–
2	A.G.	Slow fermentation	A.G.	A.G.	A.G.	A.G.	0	0	A.G.	–	+	+	–	–
12	A.G.	Slow fermentation	0	A.G.	A.G.	A.G.	0	A.G.	A.G.	–	+	+	–	–
15	A.G.	Slow fermentation	0	A.G.	A.G.	A.G.	0	A.G.	0	–	+	+	–	–

From the data in table 1, it appears that of the 38 strains the majority (27) fell into one group when judged by inability to attack sucrose; this large group could again be subdivided on the basis of dulcitol fermentation since 12 fermented this carbohydrate and 15 failed to do so. Eight of the 11 strains which attacked sucrose fermented both dulcitol and salicin. Of the other 3 sucrose fermenters, 2 fermented dulcitol but not salicin and the other the reverse.

Our cultures, then, could be divided into two groups on the basis of sucrose fermentation. The larger, which failed to attach sucrose, could be subdivided into two subgroups based on the property of attacking dulcitol. The smaller group, all strains of which fermented sucrose, was more complex in that it contained individuals which differed in their behavior in broth containing dulcitol and salicin.

The apparent failure of the organisms to utilize lactose when grown on the surface of lactose agar plates aroused our interest. Several facts were observed in this connection. The cultures grew well on the lactose plates. When true *B. coli* was seeded on the same plate with atypical strains, it readily attacked the sugar. The results then could not be attributed to poor growth or alteration of the carbohydrate. Further, the odor of the culture was different. *B. coli* was characterized by a sour odor, while the atypical type produced a strongly disagreeable ammonical one. When Durham tubes containing 1 per cent lactose are seeded with culture even after a year or more of cultivation, growth begins promptly but the indicator remains unchanged for two or more days. Acidity is first noted at the bottom of the tube and the reaction gradually changes, accompanied by a moderate gas production. Ten days or more may be required to change the color of the indicator completely. When lactose agar plates are seeded and incubated anaerobically the result is the same as when the plates are incubated in the air; the reaction of the medium is unchanged. Furthermore when certain strains of true *B. coli* of bovine origin are grown on the surface of lactose agar plates the medium is promptly acidulated. If, however, growth is artificially checked by a sharp reduction of temperature the medium becomes alkaline after a few hours, but the onset of optimum growth conditions will again inaugurate acid production. There exist then among the true colon bacilli races which under optimum conditions readily attack lactose with the formation of acid, but during retardation of growth produce sufficient alkali to shift the pH toward the alkaline side.

That perhaps there existed in our cultures of atypical colon bacilli some such balance between carbohydrate attack and alkali production seemed plausible and the following observation was designed to test this possibility.

Sugar free digest broth, pH 7.2, was distributed in amounts of 50 cc. into 100 cc. flasks. Sufficient 20 per cent lactose was added to make a concentration of 2 per cent. The flasks were inoculated in pairs with 0.2 cc. of an eighteen-hour broth culture of the organism. Half of the flasks were incubated at 38°C., and half at 22°C. The series included in table 2 contained 5 cultures of the slow lactose fermenting organisms, 1 *B. coli* which, when once it had acidulated the medium of a lactose agar plate culture, never became alkaline, and another which readily produced acid under optimum growth conditions but during retardation produced alkali. In addition the sterile medium was held for control purposes. After two-and four-day intervals the amount of lactose was quantitatively determined by means of Benedict's solution. The hydrogen ion concentration was determined and the effect of the addition of formol on pH was recorded. The results are recorded in table 2.

These observations were repeated on 3 occasions with similar results. From the data in table 2, several points are readily recognized. First, that the atypical colon bacilli utilize lactose from the start. It is true that at 38°C. the utilization is not so marked as that of typical colon types. On the other hand the amount of lactose utilized at 22°C. is comparable with that consumed by *B. coli* under the same condition. Another significant point is the effect of lactose consumption on pH. This is best illustrated in the titrations after four days of growth at 22°C. In the case of the atypical cultures, the utilization of lactose is comparable to that of colon bacilli. Nevertheless, the acidity is relatively low, varying between pH 6.8 and 7.4, whereas under the same conditions *B. coli*, while consuming no more sugar, has shifted the pH to 5.2 in one instance and 4.4 in the other. The addition of the same quantity of neutral formol to all cultures likewise reveals sharp differences. When added to the *B. coli* cultures it either had no effect (i.e., failed to reduce the final pH of 4.4) or depressed it to 4.7. The same treatment of the atypical culture revealed about the same buffer content as that possessed by the original medium, as indicated by a shift to pH 5.9. In the instance of culture brom 5-7, it required the addition of twice the amount of formol to produce this result.

TABLE 2

The utilization of lactose and acid production by various cultures

CULTURE	2 DAYS						4 DAYS					
	38°C.			22°C.			38°C.			22°C.		
	Lactose utilized	pH	pH after addition of formol	Lactose utilized	pH	pH after addition of formol	Lactose utilized	pH	pH after addition of formol	Lactose utilized	pH	pH after addition of formol
	mgm.			*mgm.*			*mgm.*			*mgm.*		
Atypical 3497-K....	6.6	6.0	5.0	7.0	7.0	6.8	11.5	6.4	5.2	8.0	7.4	5.9
Atypical A-74.......	9.0	7.8	6.0	8.4	7.4	5.8	9.5	5.9	5.4	9.3	6.8	6.0
Atypical 182........	7.7	7.2	6.0	9.7	7.0	5.8	9.0	7.8	5.9	10.0	7.4	5.8
Atypical 894........	7.4	7.0	5.6	7.0	7.0	5.8	9.0	6.8	5.4	10.9	6.8	5.9
Atypical brom 5-7..	7.6	6.9	5.9	6.8	7.4	5.9	8.2	5.6	4.4	11.0	7.0	7.0
B. coli S H 2.......	10.5	4.4	4.4	10.0	5.0	4.4	12.8	4.4	4.4	11.0	4.4	4.4
B. coli 1736*........	9.8	5.0	4.4	5.7	5.6	5.0	12.5	4.4	4.4	8.0	5.2	4.7
Uninoculated media.		7.4	5.9								7.4	5.9

* Acid during rapid growth on lactose agar plate but alkali when retarded.

There exists, then, experimental evidence that the atypical colon bacilli possess the property of utilizing lactose but at the same time produce alkali, for a time at least, in sufficient quantities to mask the acidity of fermentation.

THE IMMUNOLOGICAL PROPERTIES OF ATYPICAL COLON BACILLI

Rabbits were immunized with several strains of typical colon bacilli and with the atypical strains. In general, little relationship existed between individual strains even when drawn from the same source, as illustrated by the results recorded in table 3. The serum was prepared by immunizing a rabbit with strain 182-II and titred against other cultures identical in cultural characters and from the same plate culture.

All cultures were tested against various sera of both the typical and atypical types drawn from the same sources but no definite grouping could be established.

Stewart (1926) commented on the fact that the cultures with which he worked possessed the property of acquiring certain fermentative characteristics. This was established by observing colonies which produced buds along their borders. The organisms of the buds frequently acquired the property of fermenting the carbohydrate, an attribute not possessed by the bacteria of the original culture. This has not been true with our cultures. When buds have been formed they behave toward the environment (lactose) as did the parent colony. Many cultures were rapidly passed through lactose broth, and of these a number developed two types of colonies. One type produced acid when growing on the surface of lactose agar plates and the other behaved in all respects like the atypical culture. Certain of our strains then possessed two elements both capable of fermenting lactose but one presumably producing sufficient alkali to mask acidity and the other apparently lacking this property.

TABLE 3

Illustrating the heterogenous agglutination affinities of atypical B. coli cultures obtained from the same material

CULTURE	SERUM DILUTIONS									
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	C
Homologous 182 II	C	C	C	++++	+++	+++	++	++	+	−
182 II-0	++	+	±	−	−	−	−	−	−	−
182 II-I	C	C	+++	++	+	−	−	−	−	−
182 II-02	+	++	+	+	+	+	±	±	±	−
182 III	C	C	++	+	+	+	+	±	±	−
182 IV	++	+	−	−	−	−	−	−	−	−
182 II 40	+	++	−	−	−	−	−	−	−	−

On the other hand, once a prompt lactose fermenting type had been obtained from an atypical strain this character became fixed and rapid transfers through media containing no sugar, or rich in blood serum, never produced a reversion to the atypical form.

There were then two components in some of our strains. One, which when cultivated in a proper environment, was a true colon bacillus; the other in which the dominant factor was the atypical type.

It seemed of interest to show whether or not such a change had altered the antigenic nature of any of the forms. For this purpose 2 series of cultures were chosen, but only 1 series of experiments is reported in detail. The parent types consisted of subcultures carried on in the usual manner, the others were selected after rapid passage through broth containing lactose; those that promptly attacked lactose after several passages are referred to as Y, and those that still failed to do so under these conditions, as B. Individual rabbits were immunized with parent A, Y and B strains and the various sera tested with all the strains. Each serum was tested with both homologous and heterologous cultures. The initial titers of the various sera produced from strain 1306 are given in table 4.

The parent strain and the others possess certain antigenic properties in common as judged by agglutination. The slow lactose-fermenting B type agglutinates much better in all sera than any of the others. The character of the clumps differed sharply. B always produced the fluffy type of flocculation and the others the granular type. For a clearer understanding of these differences further experiments were necessary.

The first step consisted in the usual absorption tests. Sufficient of the heterologous strain was added to reduce greatly the agglutinin for the homologous strain. The results of this procedure are shown in table 5.

On the whole the results were unexpected. The parent strain A and the rapid lactose-fermenting type Y behave the same, each absorbing agglutinin for the other. The results with the B type, which utilized lactose slowly, appear to suggest that a new antigenic entity appeared during the course of selection, but certain

TABLE 4

The antigenic affinities of the parent (A), slow lactose fermenting (B), and prompt lactose fermenting (Y) strains

CULTURE	ANTISERUM A (PARENT)							ANTISERUM B (SLOW LACTOSE FERMENTING)							ANTISERUM Y (PROMPT LACTOSE FERMENTING)							
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	C
A	C	C	++++	++	+	+	+	++++	+++	+	+	+	+	+	C	C	++++	++	+	+	+	–
B	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	–
Y	C	C	++++	++	+	+	±	++++	+++	+	+	+	+	+	C	C	++++	+	+	+	+	–

TABLE 5

The effect of absorption of all sera with usual amounts of culture

CULTURE	ABSORBED WITH PARENT STRAIN A							ABSORBED WITH STRAIN B (SLOW ACID PRODUCTION IN LACTOSE)							ABSORBED WITH STRAIN Y (RAPID ACID PRODUCTION IN LACTOSE)						
	Dilution of serum																				
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
Serum 1306-A (parent)																					
1306-A	+	+	+	+	+	+	±	±	±	±	±	±	±	±	+	+	+	+	+	±	±
1306-B	C	C	C	C	C	C	C	++++	++++	++	+	+	+	+	C	C	C	C	++++	++++	++++
1306-Y	±	±	±	±	–	–	–	±	±	±	±	–	–	–	±	±	±	±	±	–	–
Serum 1306-B																					
1306-A	±	±	±	±	–	–	–	±	±	±	±	–	–	–	±	±	±	±	–	–	–
1306-B	C	C	C	C	++++	++++	++++	++++	+++	+	+	+	+	+	C	C	C	++++	++++	++++	++++
1306-Y	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Serum 1306-Y																					
1306-A	+	+	+	+	+	+	±	+	+	+	+	+	+	±	+	+	+	+	+	+	+
1306-B	C	C	++++	+++	+	+	+	C	C	++++	+++	+	+	+	C	C	C	C	C	C	++++
1306-Y	±	±	±	–	–	–	–	±	±	±	±	±	–	–	±	±	±	±	±	–	–

data belie this. It is recorded in table 4 that the antiserum produced by both the A (parent) and Y strain agglutinated the B culture better than the homologous strains. Therefore, both A and Y must have contained sufficient antigens similar to B to produce B antibody. That both A and Y strains possessed relatively little of such antigen can be argued from the fact that even when sufficient A and Y culture was used to reduce greatly the agglutinin for these strains the agglutinin for B remained relatively unaffected. Culture B seemed to possess the property of absorbing agglutinin for both A and Y.

TABLE 6

The effect of massive absorption of serum 1306 upon the agglutinin

SERUM	STRAIN	SERUM DILUTIONS						
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
Unabsorbed serum	A	C	C	++++	++	+	+	+
	B	C	C	C	C	C	C	C
	Y	C	C	++++	++	+	+	+
Absorbed with massive quantities of strain A	A	++	+	±	±	+	+	+
	B	++	+	+	+	+	+	+
	Y	++	+	+	+	+	+	+
Absorbed with massive quantities of strain Y	A	++	++	++	+	++	++	+
	B	++	++	++	+	++	++	+
	Y	++	++	++	++	++	++	+

If, then, strains A and Y possessed the dominant antigen for B in small quantities only it should be possible to remove agglutinin from these antisera provided large enough quantities of culture were used for absorption. This proved to be the case, as bought out in table 6.

Antiserum A was diluted 1:10 with NaCl and was repeatedly absorbed with massive quantities of either A or Y strains. As an instance, the A serum was absorbed on 6 occasions with all the bacteria obtained from the surface of 9 Blake bottles of culture Y. Another portion of A serum was absorbed with large quantities of homologous culture. The effects of such absorption on the agglutinins for B are shown in table 6.

It is evident then that the parent A strain and the active lactose fermenting Y type actually possess B antigen in sufficient quantity to produce antibody. The quantity is so small as hardly to affect the agglutination titer when the serum is absorbed by moderate quantities of A and Y cultures, but when large numbers of these organisms are employed for absorption the B agglutinin is removed. The apparent antigenic difference then is quantitative and it appears that A and Y possess similar antigens to B but in B the antigens which produce the fluffy agglutination are present in greater quantities.

In another less complete experiment both the Y and B strains developed similar antigenic qualities and agglutinated better in all sera than the parent strain.

DISCUSSION

We have described certain characteristics of a type of colon bacilli encountered in many cases of infectious diarrhea in cows. In other papers we have shown that these organisms, although frequently predominating in the feces and readily obtained from various portions of the intestinal tract of cows suffering from diarrhea, cannot be considered as the cause of the disease. Our experiments indicated that when fed to calves the organisms, either as pure cultures or fecal suspensions failed to establish themselves in the intestinal tract. In three instances cases were followed from early in the course of the disease until recovery. At the height of the attack the organisms in question were present in the feces in large numbers, during convalescence their numbers diminished, and they could no longer be found when health was restored.

From their appearance on lactose agar plates these organisms may readily be mistaken for paratyphoid bacilli especially when freshly isolated and cultivated in glucose, lactose, and sucrose. The relative tardiness with which they ferment lactose is at first deceptive although they are readily differentiated from the paratyphoids by agglutination and more complete cultural study.

We prefer to designate the organisms with which we worked as atypical colon bacilli in contra-distinction to the mutable and para types of Stewart. Our organisms invariably attacked lactose in contrast to the para type of Stewart and differed from the mutable type since it was not possible to show that they acquired new fermentative properties. On the basis of our data it seems preferable not to regard them as true mutants since we were unable to show that new characters had been developed or original properties had been lost. We have called attention to certain races of colon bacilli which readily attack lactose under optimum growth conditions and as readily produce alkali when such conditions are abruptly changed. There exists naturally a race of *B. coli* which utilizes carbohydrate under certain conditions and presumably attacks nitrogenous matter under different conditions. Our strains possess both properties. Although they utilize the carbohydrate (lactose) a little more slowly than do true *B. coli*, nevertheless during this phase they form sufficient alkali, probably ammonia, to stabilize the reaction of the media, and hence the indicator retains its original color. By rapidly passing certain strains through lactose it has been possible to obtain 2 types, one of which attacks lactose with ordinary rapidity and is essentially a true colon type and the other, which is the atypical organism. The former has not reverted to the atypical form in our hands. If, then, the atypical organism is one which differs from the typical quantitively in its ability to produce more alkali and perhaps to utilize a little less lactose, its appearance in the intestine in large numbers under abnormal conditions might be explained on the grounds of a more favorable environment during illness which facilitates the growth of this type. The lack of proper conditions might explain the apparent rarity of such organisms during health and their disappearance from the feces with a return to normal.

Certain of the immunological findings bear out the contention that members of the group differ only in degree, rather than constitutionally. The experiments reported for culture 1306 strongly support this view. Here, the parent strain and one of the derivatives which readily attacked lactose possessed identical agglutination affinities. The slowly fermenting lactose culture apparently possessed a different antigenic complex. Further experiments, however, indicated that apparent differences could be explained on quantitative grounds, so that in reality there were no appreciable antigenic differences.

The organisms are of interest to those concerned in the matters of public health since they may readily gain access to milk from the feces and at first be mistaken for paratyphoid bacilli.

SUMMARY

This paper deals with the cultural characteristics of atypical colon bacilli frequently encountered in the feces of cows suffering from intestinal disorders. In lactose agar plate cultures the organisms fail to change the reaction of the media and for this reason they may be mistaken for paratyphoid bacilli. It has been established that in broth containing lactose there is a phase in which the carbohydrate is utilized without changing the reaction of the medium, followed by a phase in which acid is slowly produced. Such organisms are not regarded as true mutants of *B. coli* since we were able to show that no new qualitative differences had developed but that apparent cultural and antigenic differences could be explained on quantitative grounds.

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THE BIOLOGY OF THE TUBERCLE BACILLUS

II. THE ASPARAGIN AND GLYCEROL METABOLISM OF THE TUBERCLE BACILLUS

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In order to understand the activities of a disease-producing organism in the host, it is desirable to determine, as nearly as possible, the metabolism of that organism. If the food of the organism were known and the method of utilization were determined, such knowledge might suggest a method of controlling the food supply in some way so as to limit the growth of the organism within the body. It is well known, for instance, that the glycerol content of a medium has a marked influence upon the growth of the tubercle bacillus, and that without nitrogenous compounds the organism will not grow. Doubtless these substances exert a similar influence within the body. How are they utilized? What products are formed? What effect have these products upon the surrounding medium? The answers to these questions may serve, by analogy, to explain some of the reactions occurring in the body of a tuberculous individual.

One method of approach to this problem is to analyze the medium on which the organisms are grown. This is done in order to show what substances are metabolized, and what changes take place. Considerable work has been done on this subject in the past, but most of the analyses have been made on media of unknown composition, such as glycerol broth, and these analyses have been chiefly of the terminal products of metabolism. In the present work, the medium was of known composition, and it was analyzed weekly in order to show the progressive utilization of the asparagin and glycerol. Is this utilization accomplished by both virulent and avirulent organisms in the same manner?

Which atom of nitrogen in the asparagin is most easily metabolized? Is the glycerol completely or partially used? What effect does the metabolism of these substances have upon the pH of the medium? To answer these questions was the object of this research.

REVIEW OF THE LITERATURE

Theobald Smith (1910) first observed that virulent and avirulent tubercle bacilli differed in their reactions in glycerol broth; the virulent organism produced a terminal acidity, while the avirulent organism produced a terminal alkalinity. He also noted that the addition of glycerol increased the growth of the virulent organism, thus indicating its utilization. In the case of *Mycobacterium tuberculosis-bovis*, however, the alkalinity of the glycerol broth offered inconclusive evidence as to the utilization of glycerol.

Kendall, Day and Walker (1914) showed that saprophytic tubercle bacilli tend to produce alkalinity when grown in glucose, mannitol or plain broth. This alkalinity, they concluded, was due to variations in the ammonia produced during metabolism. In glycerol broth, a progressive acidity was produced, which, however, might be preceded by an alkalinity. The reactions were not fixed, but varied greatly with the composition of the medium. Long and Major (1921) obtained similar results; a progressive alkalinity occurred when the organisms grew in amino acids, acid amines, and ammonium salts. The addition of glycerol, however, produced an acid reaction which was sufficiently great to mask the alkalinity. Frouin and Guillaume (1923) obtained similar results in regard to the utilization of various carbohydrates. Fosca (1924) also found a progressive acidity produced by various types of tubercle bacilli.

Weinzirl and Knapton (1927) found that virulent organisms produced progressive acidity on Long's synthetic medium while the avirulent organisms first produced marked acidity, then a regression to alkalinity. Weinzirl and Ott (unpublished data) showed that when virulent bacilli were grown on Long's synthetic medium, the elimination of asparagin did not stop the growth of the bacilli, but that no growth occurred when the glycerol was omitted. With avirulent bacilli the elimination of asparagin caused a progressive alkalinity without a previous acidity. From these results they attributed the acidity to the action on the glycerol, and the final alkalinity to the action on the asparagin.

Long and Finner (1927) showed that the growth and amount of lipin present in the bacilli is related to the amount of glycerol present in the medium, thus indicating a marked utilization of glycerol in the metabolism of the organisms.

In the work of Renfew, Bass and Johnson (1928, 1929) with human and avian tubercle bacilli grown on Long's synthetic medium it was shown that the period of growth was characterized by constant changes in the ammonia content and pH, which became comparatively constant when growth ceased. A correlation existed between the growth of the bacilli and the pH curves. The pH curve of the human strain showed progressive and permanent acidity, while that of the avian strain became acid and then alkaline.

Merrill (1930, 1931) in his work with the genus Mycobacterium cultured on broth enriched with various carbohydrates, showed that the increase in alkalinity is associated with an increase in the ammonia content of the medium, which is approximately equivalent to the increase in titratable alkalinity, and which varies directly with the pH. He concludes that if the carbohydrate molecule is attacked at all it is oxidized completely to carbon dioxide and water without the accumulation of demonstrable amounts of any intermediate products in the medium. In the repetition of this work he replaced the broth medium with a glucose synthetic medium, and found that all the carbon could be accounted for as carbon dioxide plus the carbon in the organisms and that occurring in the medium as sugar. The acidity of the medium was accounted for on a basis of the removal of ammonia from an inorganic compound, ammonium sulphate. No attempt was made to follow the course of the utilization of the nitrogen and carbon during the growth of the organisms, and conclusions were based upon the results of determinations made at the end of forty-two days.

METHODS

Medium employed. It was desirable to use a medium of the simplest composition upon which an abundant growth of the tubercle bacillus could be obtained, and in which the sources of nitrogen and carbon were limited. Many different media were tried, but the following was found to give a satisfactory growth of the tubercle bacilli: asparagin 5 grams; dipotassium phosphate (anhydrous) 1.50 grams; magnesium sulphate 1 gram; ferric chloride 0.08 gram; glycerol 50 cc.; and distilled water 1000 cc.

The ingredients were dissolved in the water in the order named, the solution titrated to a pH of 7.2, dispensed in 500 cc. flasks with 250 cc. in each, and sterilized in the autoclave at 12 pounds pressure for fifteen minutes. A small amount of precipitate of magnesium and iron phosphates formed, but was disregarded since it did not interfere with the growth.

Cultivation of organisms. The flasks of medium were inoculated with two strains of tubercle bacilli: *Mycobacterium tuberculosis*, human strain "H 37," received from the Trudeau Foundation; and *Mycobacterium tuberculosis*, human strain "H 520," an avirulent strain received from the University of British Columbia. The former grows slowly and is pathogenic; the latter grows abundantly on plain agar within twenty-four hours and will not cause tuberculosis in guinea pigs when administered intraperitoneally in 10 mgm. doses. The cultures were grown on the surface of the synthetic medium at an incubation temperature of 37°C. Small beakers were inverted over the mouths of the cotton stoppered flasks to prevent contamination. Cultures were removed for analysis on the third, seventh, tenth, and fourteenth days, and weekly thereafter for eight weeks. Uninoculated flasks of medium were subjected to the same conditions and were removed for analysis weekly in order to determine the changes due to evaporation of the medium. The organisms were removed by decantation through a filter paper, and the following determinations made upon the filtrate:

Hydrogen-ion concentration. The hydrogen-ion concentration was determined colorimetrically, using brom-thymol-blue and brom-cresol-purple as indicators. The pH was checked throughout by the potentiometer method using a quinhydrone electrode and a saturated calomel cell.

Titratable reaction. To 25 cc. portions of the medium was added 0.5 cc. of a 0.1 per cent solution of phenol red, and the culture titrated with N/40 sulphuric acid or sodium hydroxide to the same pH as the uninoculated control. This was done because an accurate endpoint could not be obtained by titrating to a color change. The results thus represent the increase in the titratable acidity or alkalinity of the culture relative to the control. This method gave quite constant results in duplicate determinations.

Ammonia. The ammonia was determined by the aeration method of Folin, the air being washed by passing through sodium hydroxide and sulphuric acid before use. The determinations were made upon 25 cc. portions of the cultures, to which from 0.5 to 1 gram of NaOH and 5 grams of NaCl were added. The ammonia was collected in N/40 sulphuric acid, and the amount of ammonia determined by back-titration using methyl red as an indicator. By determinations made upon known ammoniacal solutions, this method was found to be accurate within 0.3 cc. of N/40 reagent, or 0.12 mgm. of ammonia. Since the amount of ammonia in these cultures was found to be small, some of the results given are within the limits of experimental error. The standard solutions were checked every three weeks in order to insure their accuracy.

Amino-nitrogen. The amino nitrogen was determined by the Van Slyke method (1912, 1913, 1915). The protein was removed from the culture medium by mixing equal volumes of medium and 20 per cent trichloracetic acid. After standing an hour the protein precipitate was removed by centrifugation. Of this mixture, 2 cc., representing 1 cc. of medium, were analyzed with the micro apparatus. This method for determining amino nitrogen is accurate to 1 per cent with quantities as small as 0.5 mgm. of amino nitrogen.

Total nitrogen. The total nitrogen was determined by Gunning's modification of the macro-Kjeldahl method.

Protein nitrogen. The protein nitrogen was determined in-directly by the determination of the nitrogen remaining in the medium after the precipitation of the protein with trichloracetic acid as described under the method for amino nitrogen. The method used was the same as that for total nitrogen. The difference between the total nitrogen determinations and the determinations following the precipitation of the protein indicated the amount of protein nitrogen. This method allowed for some error due to the fact that some of the protein was probably converted into soluble acid albuminates and was not removed from the solution.

Rest Nitrogen. This value was calculated by subtracting the combined amino nitrogen and protein nitrogen from the total nitrogen. It represents chiefly amino nitrogen from the asparagin, with ammonia, proteoses, basic amines, or any other nitrogenous substances which may be produced by the organisms during their growth.

Glycerol. The glycerol was determined by method I, in the Methods of Analysis of the Association of Official Agricultural Chemists (1925) for the official determination of glycerol in dry wines. In this method 100 cc. of the medium were used for analysis, the glycerol being determined by evaporation and subsequent extraction with boiling alcohol and an alcohol-ether mixture. The glycerol was then determined gravimetrically. This method allows for a considerable error due to the fact that some glycerol is volatile at 90° to 100°C., although milk of lime was added to decrease this volatility. The results, however, are sufficiently accurate to show the course of the glycerol utilization.

The results obtained by all of the above methods were checked by duplicate determinations and the two figures averaged for use in establishing the curves.

RESULTS

In an attempt to follow the reaction curves produced by the tubercle bacilli in a simplified medium of known composition, and to show the relationship between the reaction curves and the metabolism of the asparagin and glycerol by the organisms, quantitative methods were relied upon. The metabolism of the asparagin was followed by four nitrogen determinations: (1)

nitrogen in the form of free or combined ammonia; (2) amino nitrogen; (3) protein nitrogen, and (4) total nitrogen. By comparison of these results, the amount of asparagin utilized can be determined, and to a certain extent its manner of utilization. It was assumed that the nitrogen which was not in the medium had been incorporated into the bodies of the organisms. It is possible, but not very probable, that a small amount of nitrogen may have escaped into the air.

The glycerol metabolism was followed by direct quantitative determination of the amount of glycerol in the medium, the decrease in glycerol content indicating its utilization by the organism. Whether or not such utilization was complete was difficult to prove, but it was thought that if there were partial oxidation of the glycerol to organic acids, as some investigators (Long, Campbell and Smith (1922)) have supposed, lactic and acetic acids would, presumably, be formed to some extent. In order to determine whether or not these acids were formed, the ferric chloride test for lactic acid were tried using ether extraction, and the U. S. P. tests for acetic acid and acetates. Satisfactory positive tests were not obtained for either of these acids, which would seem to indicate that if partial oxidation of the glycerol to these organic acids took place, it was only to a very slight extent.

The titratable reaction was determined to see whether or not there was a direct relationship between the pH curves and the titratable acidity or alkalinity, and whether or not the titratable alkalinity could be accounted for by the ammonia production.

The data obtained for *Mycobacterium tuberculosis,* human strain H 37, are presented in table 1.

Table 1 indicates the following: (1) the pH shows a progressive rise of acidity from pH 7.2 to 5.2 which has been paralleled throughout by the titratable reaction; (2) The ammonia formed has been slight, but the maximum amount seems to occur at the time of maximum growth; (3) the amino nitrogen, total nitrogen, and rest nitrogen show, in general, a decrease; (4) the protein does not appear in appreciable quantity until the fifth week; (5) the glycerol has been practically all utilized at the end of the ten weeks' incubation.

Table 2 shows the quantitative data obtained during the growth

TABLE 1

Giving data obtained for Mycobacterium tuberculosis, human type 37

AGE	pH	TITRATABLE REACTION	AMMONIA	AMINO N	TOTAL N	PROTEIN N	REST N	GLYCEROL
days		*cc. N/40 per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*grams per 100 cc.*
0	7.2	0	0	46.5	88.65		42.15	4.4
3	7.1	0	0	46.32	88.63		42.33	4.4
7	7.1	0.65 NaOH	0.37	46.60	88.18		41.58	
10	7.0	0	0.93	46.14	86.47		40.33	4.1
14	7.0	0.436 NaOH	0.55	42.25	87.40		45.15	3.9
21	7.4	8.12 H_2SO_4	7.48	40.10	70.60	0.92	29.58	4.2
28	7.1	8.72 NaOH	1.48	36.20	52.25		16.05	4.0
35	6.4	10.47 NaOH	2.04	41.40	58.78	10.38	7.00	3.65
42	6.3	10.9 NaOH	12.2	34.30	58.16	10.67	13.19	3.09
49	6.2	8.72 NaOH	2.04	13.32	25.31	3.18	8.86	2.75
56	5.8	9.81 NaOH	1.3	3.31	19.68	4.38	11.99	1.99
63	5.6	10.03 NaOH	1.09	3.30	22.43	3.73	15.4	1.26
70	5.2	17.23 NaOH	1.09	2.75	22.34	2.04	17.55	0.52

TABLE 2

Giving data obtained for Mycobacterium tuberculosis, human type 520

AGE	pH	TITRATABLE REACTION	AMMONIA	AMINO N	TOTAL N	PROTEIN N	REST N	GLYCEROL
days		*cc. N/40 per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*grams per 100 cc.*
0	7.2	0	0	46.5	88.65		42.15	4.4
3	6.9	4.8 NaOH	0	42.25	79.94		37.69	4.28
7	6.6	7.85 NaOH	0.92	38.92	61.27		22.35	
10	6.2	15.5 NaOH	0.55	40.03	60.34		20.31	3.5
14	5.6	16.8 NaOH	1.11	38.92	58.47	1.79	17.76	3.7
21	5.8	11.34 NaOH	1.2	27.46	39.8	3.9	8.44	2.8
28	6.2	4.36 NaOH	0.9	4.9	22.69	14.29	3.5	1.1
35	6.9	2.0 H_2SO_4	0.7	0.5	22.38	7.7	14.8	
42	6.9	2.18 H_2SO_4	0.37		22.69	6.79	15.9	
49	7.0	1.87 H_2SO_4	0.55	1.1	27.49	10.69	15.7	
56	7.1	2.5 H_2SO_4	0.46		27.18	11.88	15.3	
63	7.1	2.96 H_2SO_4	0.37	1.65	26.56	10.66	14.25	
70	7.2	3.28 H_2SO_4	0.46	3.85	28.43	10.63	13.95	

of *Mycobacterium tuberculosis*, human type 520 (saprophytic), upon the synthetic medium. In general, the results show that the metabolism of the saprophytic H 520 closely parallels that of the virulent H 37. The chief differences are: (1) the changes occur more rapidly with the saprophytic organism than with the virulent organism; (2) the pH of the saprophytic organism becomes acid and then alkaline while that of the virulent organism remains acid.

Table 3 shows the quantitative data obtained from the analysis of the medium in uninoculated control flasks exposed to the same incubational conditions as those of the inoculated flasks. These determinations serve merely as checks upon those of tables 1 and 2.

TABLE 3

Giving data obtained for the control flasks of uninoculated medium

AGE	pH	TITRATABLE REACTION	NH_3	AMINO N	TOTAL N	PROTEIN N	REST N	GLYCEROL
days		*cc. N/40 per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*grams per 100 cc.*
0	7.2			46.5	88.65		42.15	4.4
3	7.2			46.5	88.65		42.15	4.4
7	7.2			46.3	88.65		42.35	
10	7.2			46.7	88.65		41.95	4.0
14	7.2			46.3	89.27		42.97	4.3
21	7.1			47.3	89.27		41.97	4.4
28	7.1			46.75	89.89		43.14	4.3
35	7.1			47.0	90.51		43.51	
42	7.1			47.4	90.2		42.8	4.6
49	7.0			47.7	89.74		42.04	4.14
56	7.0			47.7	89.0		42.7	4.6
63	7.0			47.7	90.6		42.9	4.24
70	7.0			47.7	89.37		41.67	4.5

A graphic representation of the changes produced by the organisms is shown in figures 1 to 8.

DISCUSSION

In an interpretation of the results based upon analytic determinations of substances in a culture medium, it is necessary to remember the error contingent upon the effect of the organisms' excretions and lysis. Since the organism maintains a balance between the food ingested and the waste products excreted, the medium contains not only the unused food substances placed in

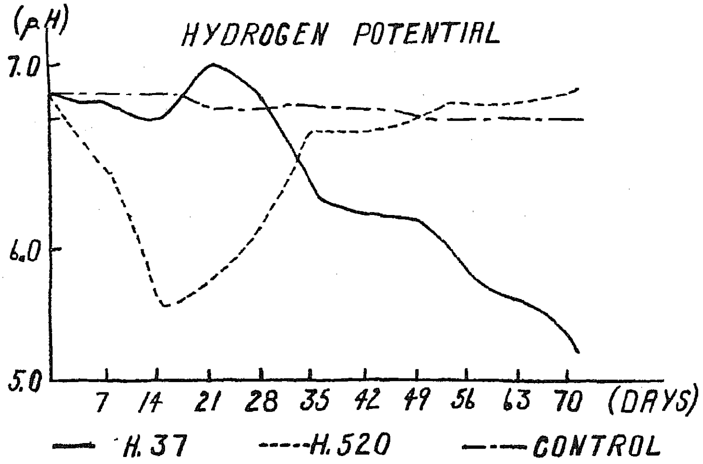
(pH)
HYDROGEN POTENTIAL
7.0
6.0
5.0
7
14
21
28
35
42
49
56
63
70
(DAYS)
H. 37
H. 520
CONTROL

FIG. 1

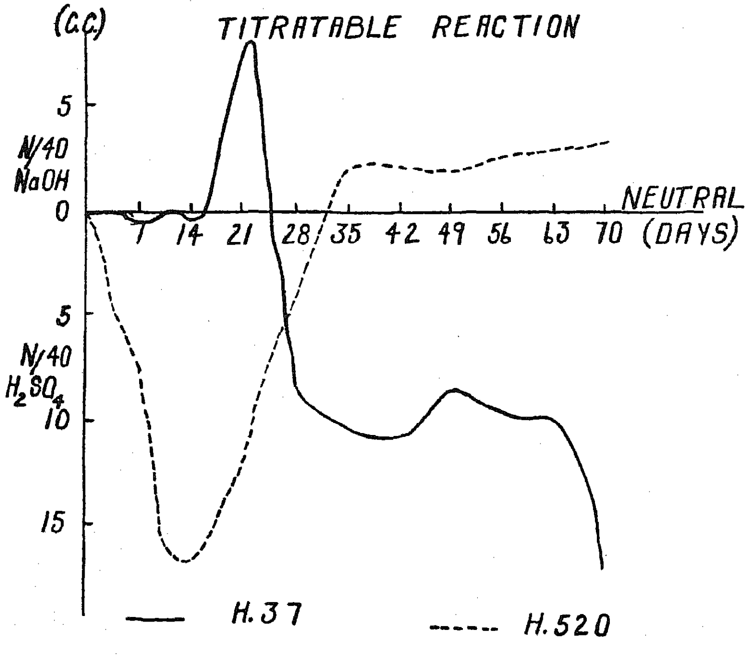
(C.C.)
TITRATABLE REACTION
5
N/40 NaOH
0
NEUTRAL
7
14
21
28
35
42
49
56
63
70
(DAYS)
5
N/40 H₂SO₄
10
15
H. 37
H. 520

FIG. 2

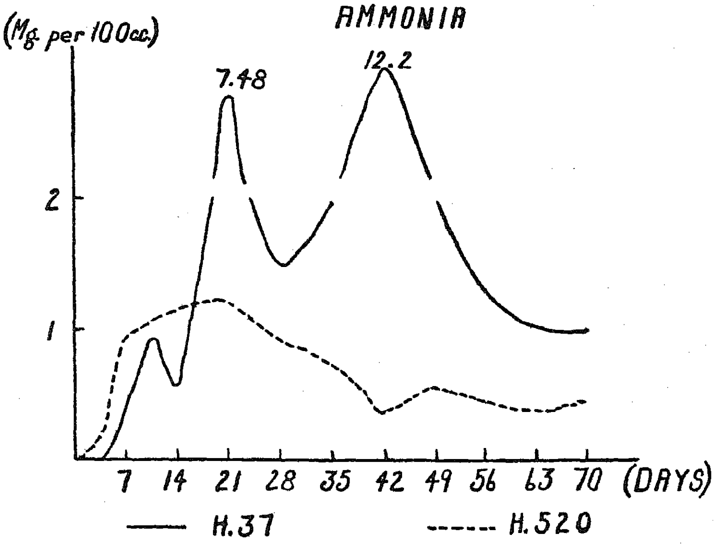
AMMONIA
(Mg. per 100cc.)
7.48
12.2
2
1
7
14
21
28
35
42
49
56
63
70
(DAYS)
H.37
H.520

FIG. 3

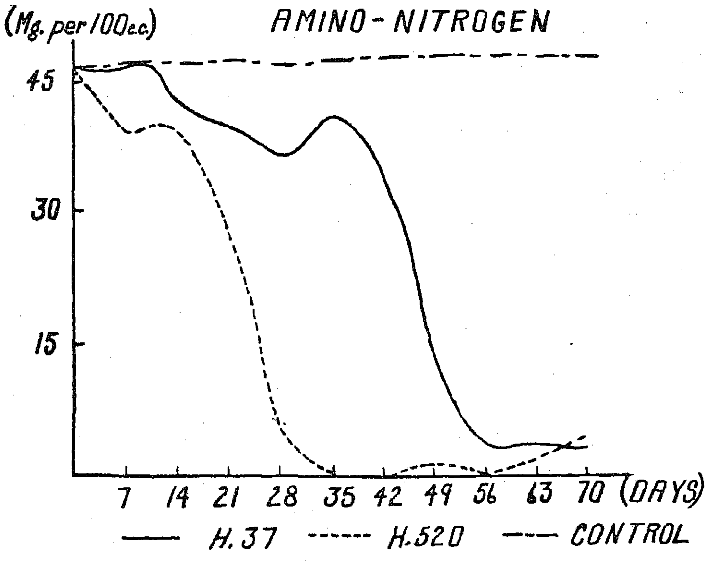
AMINO-NITROGEN
(Mg. per 100c.c.)
45
30
15
7
14
21
28
35
42
49
56
63
70
(DAYS)
H.37
H.520
CONTROL

FIG. 4

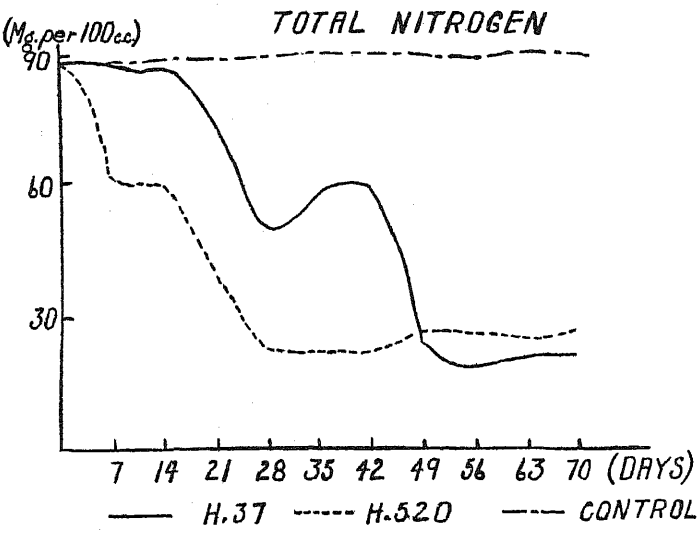
TOTAL NITROGEN
(Mg. per 100cc.)
90
60
30
7
14
21
28
35
42
49
56
63
70
(DAYS)
H.37
H.520
CONTROL

FIG. 5

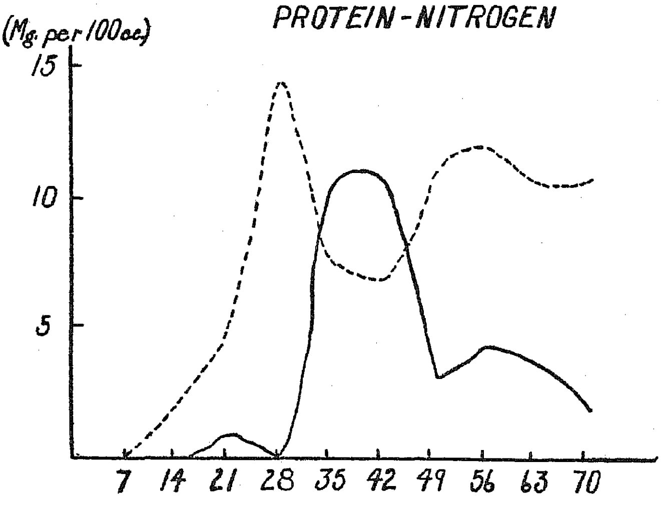
PROTEIN-NITROGEN
(Mg. per 100cc.)
15
10
5
7
14
21
28
35
42
49
56
63
70
H.37
H.520

FIG. 6

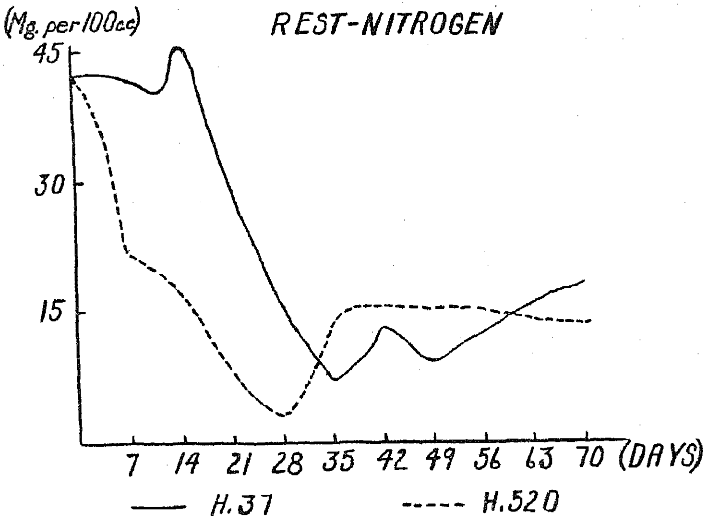
REST-NITROGEN
(Mg. per 100cc)
45
30
15
7
14
21
28
35
42
49
56
63
70
(DAYS)
—— H.37
----- H.520

FIG. 7

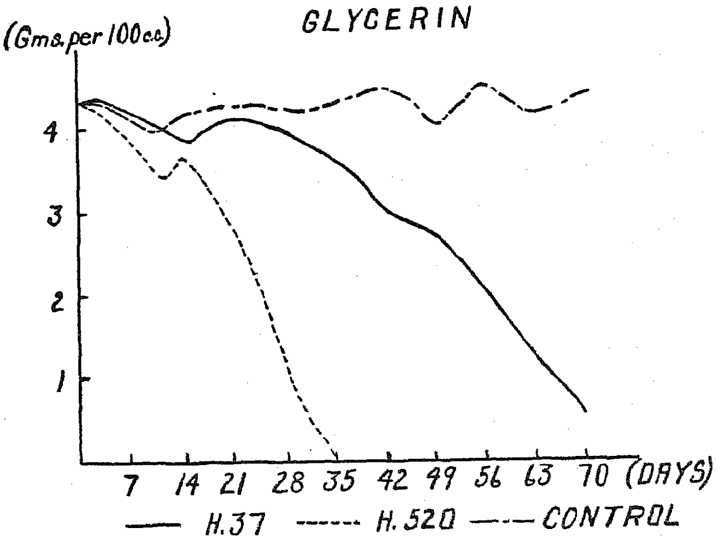
GLYCERIN
(Gms. per 100cc)
4
3
2
1
7
14
21
28
35
42
49
56
63
70
(DAYS)
—— H.37
------ H.520
—-— CONTROL

FIG. 8

it originally, but also the excretory and lytic products of the organism. If the organism metabolised its food completely to carbon dioxide and water, such excretion products would have little effect upon these determinations. But apparently it does not do this, as evidenced by the fact that protein and other nitrogenous compounds which must have come from the organism, since there were none in the original medium, appear after three or four weeks' growth. This protein may be due in part to lysis of the organism, and in part to its solution. It seems probable that the organism might eliminate not only protein but also other complex nitrogenous compounds, such as proteoses, peptones and amines. On this account, in a cautious interpretation of results based upon these determinations, no rigid lines can be drawn to show just how much of the materials have been utilized and in what manner such utilization has taken place.

The pH curves, shown in figure 1, agree closely with those given by other investigators. A progressive acidity was noted in the cultures of the virulent H 37, and acidity which returned to alkalinity in the cultures of the virulent H 520. The alkalinity shown at the third week by the pH curve of H 37 is also shown by the titratable reaction and an increase in the ammonia content of the medium. No cause could be determined for this alkalinity. Both Theobald Smith (1910) and Merrill (1930) report that in some cultures of virulent tubercle bacilli they have found an initial alkalinity occurring before the progressive acidity. The pH curve for the avirulent bacilli (H 520) progressed to an acidity of 5.6 and then returned to an alkalinity of 7.2, the same as that of the initial uninoculated medium. The pH of the uninoculated control progressed from 7.2 to 7.0 during the duration of the experiment. This change was possibly due to absorption of carbon dioxide from the air, and to evaporation of the medium with a resultant change in its buffering power.

The titratable reaction of the culture medium, expressed in cubic centimeters of N/40 acid or alkali paralleled the pH closely, as is shown by figure 2. The alkalinity occurring in the last few week's growth of the avirulent organisms (H 520), as expressed by the titratable alkalinity, may be due to the fact that the medium was titrated to the same reaction as the control. Since the latter became acid from 7.2 to 7.0 in the last seven weeks of incubation, the addition of acid was required to make the reaction of the culture and control identical, as the pH of the culture returned to the initial pH.

The ammonia determinations, figure 3, showed a progressive increase in the amount of ammonia present in the medium up to the time of maximum growth of the organisms as indicated by their bulk. The increase was marked in H 520 up to the third week, following which there was a gradual recession of ammonia. With H37, the increase was more gradual and reached a maximum about the sixth or seventh week, following which there was a marked recession. This decrease in the ammonia content may have been due to the loss of ammonia from the medium into the air, and also to the utilization of the ammonia by the organisms. Since asparagin was the only source of nitrogen, and thus of ammonia in the medium, the ammonia must have come from the breaking down of the asparagin by the organisms, probably by enzymic action in the medium or by absorption of the whole molecule and resultant excretion of the ammonia. As will be shown below, the asparagin was almost completely broken down during the progress of the experiment. Since more nitrogen would be essential to the further growth of the organisms, it seems likely that the ammonia and other nitrogenous excretory and lytic products may have served as partial sources of nitrogen for further growth. The cause of the two extreme variations in the ammonia content of the H 37 medium can only be determined by further experiments. The ammonia curve for H 520 was at its highest point at approximately the same time that the pH was the most acid, and decreased as the pH became alkaline, indicating that the alkalinity was not due to ammonia formation.

The amino nitrogen determinations, figure 4, made upon the medium after the precipitation of the protein with trichloracetic acid, show practically complete utilization of the amino nitrogen by both organisms. The virulent H 37 organisms metabolized the nitrogen only slightly during the first six weeks, but from the sixth to the eighth week there was marked utilization as shown by the decrease in the amino nitrogen content, until only a small amount was left in the medium. The avirulent H 520 metabolized the amino nitrogen slightly during the first and second weeks, but a marked decrease in the amino nitrogen content occurred in the third, fourth, and fifth weeks when no nitrogen could be detected by this method. At the ninth and tenth weeks a slight amount of amino nitrogen was noted. This was probably due to the lysis of some of the organisms and to the partial hydrolysis of their proteins. The control flasks showed an increase of approximately 1 mgm. of amino nitrogen per 100 cc. of the medium during the period of incubation. This increase was presumably due to evaporation of the medium.

The curves shown in figure 5 indicate the progressive decrease in the total nitrogen content of the medium. This decrease is approximately the same with both organisms, although it occurs much more rapidly with H 520 than with H 37. The lowest point is reached at the fourth week in the H 520 cultures, and not until the eighth week in the H37 cultures. Up to these points the greatest amount of nitrogen was probably in the form of asparagin, but from this time on it seems likely that the largest amount of nitrogen was in the form of protein nitrogen and other nitrogenous excretory and lytic products of the organisms.

The protein nitrogen (fig. 6), appeared between the third and fifth weeks in the culture medium of the H 37 organisms, and about the second week in that of the H 520 organisms. In both, the protein nitrogen increased rapidly and then decreased, the decrease being more marked in the case of H 37. This may possibly be explained by the fact that the H 37 medium became quite acid, which would tend to favor proteolysis, while the H 520 medium returned to approximately neutrality, which would probably retard the proteolytic process.

The rest nitrogen (fig. 7), consisted chiefly of the imino nitrogen of the asparagin in the early part of the experiment, and of the nitrogenous excretory and lytic products of the organisms in the latter part. In the H 37 medium the decrease in the rest nitrogen (chiefly imino nitrogen) was approximately the same as the decrease in the amino nitrogen for the first two weeks. During the third and fourth weeks, however, the decrease was very marked until at the end of the fifth week it reached the lowest point, while the amino nitrogen had scarcely decreased at all. At this same time the protein nitrogen was approaching a maximum, and it would appear likely that the imino nitrogen may be entirely metabolized and that the remaining nitrogen figured here was due to other nitrogenous compounds. This same relationship existed in the avirulent H 520 medium, although the changes occurred more quickly than in the H 37 medium. These results would seem to indicate that the organisms utilized the imino nitrogen more readily than the amino nitrogen.

In a consideration of the above results with respect to the pH reactions produced in the medium, several possibilities presented themselves. The organisms could produce an acidity (1) by removing an alkaline substance from the medium, (2) by the partial utilization of a neutral compound leaving an acid one, (3) by the excretion of an acidic compound, or (4) by some combination of these; the processes reversed would cause an alkalinity. The pH changes may have been due to the metabolism of the inorganic salts, glycerol, and asparagin in this medium.

No determinations were made of the inorganic constituents of the medium, hence the effect of their metabolism upon the medium cannot be stated.

The glycerol metabolism may account in part for the acidity produced in the medium. Long (1922) believes that this is the case, but Merrill (1930) believes that the glycerol as well as other carbohydrates are completely metabolised to carbon dioxide and water. Our results seem to favor the latter view, since the glycerol as such was completely removed from the medium and no tests were obtained for lactic and acetic acids. However, it is possible that other acids or acidic compounds were produced from the glycerol.

The acidity may be due to the removal of the basic nitrogenous radicals from the neutral asparagin by enzymic or hydrolytic processes. It seems likely that this is the case and that the carbon of the asparagin is not readily utilized, since the virulent organism will not grow with asparagin as the only source of carbon. If the acidity were produced in this manner, leaving an acidic organic compound in the medium, the return of the pH to neutrality in the medium of the avirulent organism might be explained by the subsequent utilization of this compound as a source of carbon. Thus it seems likely that in our medium the pH curve may have been to a large extent dependent upon the metabolism of the asparagin.

CONCLUSIONS

1. In this work two strains of tubercle bacilli were employed, namely, a virulent H 37 and avirulent H 520. These seemed to be alike in their metabolism on the synthetic medium employed except that the avirulent H 520 bacilli grew more rapidly and produced a terminal alkalinity, while the virulent H 37 grew more slowly and produced a terminal acidity.
2. The titratable reaction and the pH curves closely paralleled each other throughout the course of the experiment. Apparently there was no relationship between the titratable alkalinity and the ammonia.
3. The ammonia content of the medium in the case of both organisms was small, but showed a maximum content at the time of greatest growth, followed by a recession. This would indicate that the alkalinity produced in the H 520 medium was not due to ammonia.
4. The nitrogen determinations showed that practically all of the nitrogen of the asparagin was utilized by both organisms. Apparently the organisms utilized the imino nitrogen of the asparagin more readily than the amino nitrogen.
5. The glycerol was practically completely utilized by both organisms, the decrease in the amount in the medium occurring much more rapidly with the H 520 than with the H 37. It appeared likely that the utilization was complete and that the glycerol was not partially oxidized to organic acids.
6. The acidity produced by the organisms in the medium seemed to a considerable extent to be due to the metabolism of the asparagin. The terminal alkalinity produced by the avirulent organism may be explained by the assumption that there oc-

curred further utilization of acidic compounds produced from the asparagin.

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OBSERVATIONS ON BACILLUS COAGULANS[1]

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Bacillus coagulans was described at the Iowa Agricultural Experiment Station (Hammer (1915)) as the cause of an outbreak of coagulation in evaporated milk packed by an Iowa condensery.

Cordes (1928) found *B. coagulans* responsible for an outbreak of "flat-sours" in evaporated milk. The cans from which the organism was secured had been subjected to 114.4°C. (238°F.) for twenty minutes in a batch sterilizer. However, milk cultures of the organism were killed when exposed in an autoclave to a temperature of 111.7° to 112.8°C. (233° to 235°F.) for fifteen minutes, the milk itself reaching 112.2°C. (234°F.), being between 100° and 112.2°C. (212° and 234°F.) for six minutes, and between 107.2° and 112.2°C. (225° and 234°F.) for four minutes.

B. coagulans was recently found to be the cause of an outbreak of coagulation in evaporated milk packed by a condensery in a neighboring state. A number of the cultures isolated were studied in order to (1) check and enlarge the description of the organism, and (2) secure additional information on the changes it produces in evaporated milk.

GENERAL CHARACTERS OF OUTBREAKS OF COAGULATION IN EVAPORATED MILK DUE TO B. COAGULANS

In 1915 an outbreak of coagulation in evaporated milk occurred in an Iowa condensery and extended over a period of several months, with the May and June milk showing the highest percentage of spoilage. *B. coagulans* was isolated from the spoiled milk. At the time coagulation was first encountered the heat treatments used varied with the milk, but were approximately 110° to 111.7°C. (230° to 233°F) for from thirty to thirty-six minutes. The exposures were increased, and although the percentage of spoilage decreased, the loss was still sufficient to be of considerable consequence. It was reasonably certain that some of the milk which spoiled was heated to 113.3°C. (236°F.) for thirty-six minutes. The milk which spoiled had been held at the condensery for at least ten days before it was sent out, and was then apparently normal, so coagulation did not occur rapidly. Most of the abnormal cans were firmly curdled, but a few showed a soft, flaky curd with considerable whey. The spoiled milk had a sweetish, cheesy odor, resembling to some extent the odor of Swiss cheese, and a sour, cheesy flavor. The odor and flavor were not disagreeable and there was no suggestion of putrefaction. Acid determinations on milk that coagulated during the outbreak, and on milk that coagulated following the inoculation of a pure culture of the organism, showed that the abnormal condition was accompanied by a considerable increase in acidity.

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[1] Journal Paper Number B1 of the Iowa Agricultural Experiment Station.

The outbreak supplying the spoiled evaporated milk from which *B. coagulans* was recently isolated occurred during the abnormally hot, dry weather of the summer of 1930. The outreak extended over a period of several weeks. The percentage of cans that spoiled varied greatly from one batch to another and, in one instance, was estimated by the plant managers to be as high as 60 per cent. The typical spoiled milk was firmly curdled, but in some cans the coagulation was less complete, due presumably to a less extensive growth of the organisms. The odor of the spoiled milk was distinctly cheesy, with a definite suggestion of acidity, and the flavor was sour and cheesy. At the condensery experiencing the difficulty it was noted that the acidity of the normal milk was 0.48 per cent while that of the spoiled milk was 1.05 per cent, calculated as lactic acid. Although the odor and flavor of the spoiled milk were decidedly abnormal, they were not suggestive of putrefaction; in a hard cheese they would have been quite acceptable.

The spoilage began with batches of milk subjected to a heat treatment which had been satisfactory over a long period. With the appearance of the defect the temperature was increased somewhat, but coagulation of the milk continued. The plant reported considerable spoilage with one batch which was run through a standard heater for twelve minutes up to 97.8°C. (208°F.), a variable heater for twelve minutes at 97.8°C. (208°F.), and a cooker for fifteen minutes at 117.8°C. (244°F.).

EXPERIMENTAL

The cultures studied. As a rule the coagulated evaporated milk contained a sufficient number of viable organisms so that direct plating on beef-infusion agar gave a distribution of colonies satisfactory for isolation. With one rather old sample it was necessary to inoculate a beef-infusion agar slant with a small amount of the coagulated milk and then prepare plates from the growth developing on the surface of the slant. All of the cultures secured by these procedures were replated to insure their purity.

The observations made involved nine cultures isolated from seven lots of spoiled milk; cultures 1, 2, 3, 4, 5, and 9 were all from different lots while cultures 6, 7, and 8 were from the same lot. Eight of the cultures were isolated about one month after the milk had coagulated while one (culture 9) was isolated about five months after the milk had coagulated.

A detailed study of the morphology, cultural characteristics, and biochemical features of the nine cultures confirmed the original description of the organism. This description, enlarged by recent findings, is as follows.

DESCRIPTION OF BACILLUS COAGULANS

Morphology

Form and size. Rods; 0.5 to 0.7 by 1.6 to 7.1 microns when grown on beef-infusion agar (twenty-four hours at 37°C.); somewhat smaller when grown in milk (forty-eight hours at 37°C.).

Arrangement. Singly and in short chains.

Motility. Motile; flagellation peritrichous.

Staining reactions. Gram-positive in young cultures, often with distinct granulation; commonly Gram-negative in old cultures although a few Gram-positive cells sometimes persisted.

Spore formation. In old beef-infusion agar slant cultures and in coagulated evaporated milk some cells contained spores. Preparations made from agar or milk cultures grown under various incubation conditions regularly showed spores in less than half of the cells. The spores were small, round, did not bulge the cells and were sub-terminal.

Cultural characteristics

Agar slant. Beef-infusion and whey agars showed abundant, echinulate, white, non-viscid, shiny growth after two to three days at 37°C. Growth less abundant on standard agar.

Agar stab. Beef-infusion and whey agars showed heavy, white, non-viscid, surface growth with some growth along the line of inoculation after two to three days at 37°C.

Agar colony. After two to three days at 37°C. surface colonies on beef-infusion agar were shiny, white, non-viscid, round, about 1 to 2 mm. in diameter, with entire edge. Sub-surface colonies were round to oval, white, non-viscid, and smaller than the surface colonies.

Gelatin stab. On whey gelatin at 37°C. growth occurred; gelatin not liquefied.

Broth. Turbidity with sediment.

Potato. Dirty white, shiny, non-viscid, spreading growth.

Litmus milk. Litmus milk was reduced. Reduction was followed by coagulation and appearance of red band at top of milk. Red band increased in depth, curd contracted, expressing small amount of whey. No apparent proteolysis. Coagulation in four to eight days at 37°C. and in two to four days at 50°C.

Biochemical features

Indol. Not produced.

Nitrates. Not reduced.

Action on carbohydrates and alcohols. Glycerol, glucose, levulose, galactose, lactose, maltose, sucrose, salicin, raffinose, dextrin, and soluble starch fermented with the production of acid but no gas; arabinose, adonitol, dulcitol, inositol, mannitol, sorbitol, and inulin not fermented; starch hydrolyzed.

Oxygen relationship. Organism facultative; grew well aerobically.

Growth temperatures. Grew well between 37° and 55°C.; poorly, if at all, at 20°C.

Numbers of organisms in the spoiled evaporated milk. a. Milk which coagulated at the condensery. Several cans of evaporated milk, which had been processed during August, 1930, were sent to the laboratory for examination. Upon their arrival, October 15, 1930, they were placed in the cooler at about 7°C. and held there until plated on November 8.

After thorough shaking, 1 ml. of the contents of each can was removed, and dilutions plated on beef-infusion agar; the plates were incubated four days at 37°C. The results obtained are given in table 1.

The plate counts on the milk held for considerable periods after coagulation at the condensery varied from 7,000 to 77,000 per milliliter and thus indicate that there should be little difficulty in securing *B. coagulans* from spoiled milk, even if the milk is not examined soon after spoilage occurs.

TABLE 1

Plate counts on evaporated milk which coagulated at the condensery

DATE PROCESSED (AUGUST, 1930)	AGE WHEN PLATED	PLATE COUNT PER MILLILITER
	days	
7	93	23,000
7	93	22,000
7	93	77,000
7	93	31,000
20	80	58,000
22	78	7,000
25	75	11,000

b. Milk which coagulated following inoculation with B. coagulans. Cans of normal evaporated milk were inoculated with *B. coagulans* and incubated at 37° or 50°C. for varying periods of time. Beef-infusion agar cultures grown at 37°C. for forty-eight hours were used for inoculation. This was carried out by covering a small area on an end of a can with concentrated HCl, flooding the area with solder, punching a hole in the center of the area with a nail which had been heated to redness and cooled, and then adding the organisms with a needle; after inoculation the can was immediately resealed with solder and shaken thoroughly to distribute the organisms. The controls were punched and resealed, while unopened cans were also used in order to check the sterility of the original milk. After incubation under varying conditions plate counts were made on the coagulated milk, using beef infusion agar and an incubation of four days at 37°C. The data secured are given in table 2.

TABLE 2

Plate counts on evaporated milk which coagulated following inoculation with B. coagulans

CULTURE USED TO INOCULATE	TEMPERATURE OF INCUBATION	TIME TO COAGULATE	PERIOD OF INCUBATION	PLATE COUNT PER MILLILITER
	°*C.*	*days*	*days*	
1	37	7	8	41,000,000
1	37	4	6	100,000,000
2	37	8	8	51,000,000
2	37	5	18	220,000
3	37	8	8	44,000,000
3	37	5	50	20,000
4	37	10	10	36,000,000
5	37	10	10	33,000,000
5	37	10	14	21,000,000
5	37	7	21	220,000*
5	50	4	50	300
6	37	10	10	33,000,000
6	37	8	14	29,000,000
6	37	7	39	110,000
6	50	4	39	21,000
7	37	6	18	12,000,000
7	50	3	50	400
8	37	7	21	200,000†
8	50	4	39	25,000

* Stored twenty-nine days at room temperature following period of incubation.

† Stored eighteen days at room temperature following period of incubation.

The results indicate that at 37°C. the number of organisms present soon after the milk coagulated was large (up to 100,000,000 per milliliter), and that continued incubation resulted in a decrease in the count. None of the cans incubated at 50°C. were plated soon after coagulation, but following the long incubation period the numbers were comparatively small. In general, the results indicate that there should be little difficulty in isolating *B. coagulans* from spoiled milk even if the examination is delayed. The time required for the inoculated milk to coagulate firmly varied from four to ten days at 37°C. and from three to four days at 50°C.

Total and volatile acidities of the spoiled evaporated milk. The total and volatile acidities were studied on a number of samples of the spoiled evaporated milk. The total acidity was determined by titrating a 20-gram sample with 0.1 N NaOH, using phenolphthalein as an indicator, and calculating the acidity as lactic acid. The volatile acidity was determined by steam distilling a 250-gram sample of the milk after acidifying with 15 ml. N H_2SO_4; 1 liter of distillate was collected, a 100 ml. aliquot titrated with 0.1 N NaOH using phenolphthalein as an indicator, and the milli-

TABLE 3

Total and volatile acidities of evaporated milk which coagulated at the condensery

TREATMENT OF CANS				TOTAL ACID AS LACTIC	VOLATILE ACID: 0.1 N NaOH REQUIRED TO NEUTRALIZE 1,000 ML. STEAM DISTILLATE FROM 250 GRAMS MILK
Date processed (August, 1930)	Period of holding				
	At condensery	At laboratory			
	days	*days*	*°C.*	*per cent*	*ml.*
7	60	33	7	1.06	33
7	60	33	7	1.06	33
7	60	33	7	1.03	35
7	60	33	7	1.03	27
20	47	33	7	0.90	22
22	45	33	7	0.97	37
25	42	33	7	1.06	24
27	154	41	21	0.92	38
27	154	41	21	0.94	40
27	154	41	21	0.85	28
27	154	41	21	0.97	
27	154	41	21	0.48*	
27	154	41	21	0.49*	6
27	154	41	21	0.43*	
27	154	41	21	0.42*	
27	154	41	21	0.42*	

* Normal milk.

liters of 0.1 N NaOH that would have been required for the liter of distillate then calculated. The results obtained on milk which spoiled at the condensery are given in table 3, and those obtained on milk inoculated with *B. coagulans* in table 4.

TABLE 4

Total and volatile acidities of evaporated milk which coagulated following inoculation with B. coagulans

CULTURE USED TO INOCULATE	TEMPERATURE OF INCUBATION	TIME TO COAGULATE	PERIOD OF INCUBATION	TOTAL ACID AS LACTIC	VOLATILE ACID: 0.1 N NaOH REQUIRED TO NEUTRALIZE 1,000 ML. STEAM DISTILLATE FROM 250 GRAMS MILK
	°C.	*days*	*days*	*per cent*	*ml.*
1	37	4	6	1.06	
1	37	4	31	1.07	22
1	37	7	8	1.30	25
2	37	3	31	1.23	16
2	37	8	8	0.97	22
3	37	5	50	1.23	
3	37	4	31	1.28	18
3	37	8	8	1.05	28
4	37	4	31	1.15	19
4	37	10	10	0.95	23
5	37	10	14	0.86	
5	37	7	20	1.13	
5	37	10	10	0.87	20
5	50	4	50	0.95	
6	37	8	14	0.94	
6	37	10	10	0.93	21
7	50	3	50	0.98	
Unopened control.	37		10	0.49	
Unopened control.	37		31	0.43	
Unopened control.	37		50	0.48	
Opened and resealed control...	37		31	0.45	

The data in table 3 show that spoilage was accompanied by a large increase in total acidity, the abnormal milk showing acidities from 0.85 to 1.06 per cent while the normal product showed acidities from 0.42 to 0.49 per cent. The volatile acidities were also greatly increased, showing values from 22 to 40 while the one determination on a normal product gave a value of 6. The results in table 4 are in general agreement with those in table 3, the total acidities of the spoiled milk ranging from 0.86 to 1.30 per cent and of the normal milk from 0.43 to 0.49 per cent while the volatile acidities of the spoiled milk ranged from 16 to 28. The inoculated cans held at 37°C. required from three to ten days to coagulate while the two cans held at 50°C. required three and four days, respectively.

The non-volatile acid in the spoiled evaporated milk. The results reported on total and volatile acidities in the spoiled evaporated milk indicate that a large percentage of the acid is non-volatile. Zinc salts were accordingly prepared from the residues remaining after the steam distillations, using the method outlined by Hammer (1920). The results obtained in the study of these salts are given in table 5; samples 1, 2 and 3 were from milk which coagulated at the condensery while 4 and 5 were from milk which coagulated following inoculation.

TABLE 5

Data on zinc salts prepared from the non-volatile acid in the spoiled evaporated milk

SAMPLE	WATER OF CRYSTALLIZATION IN ZINC SALT			ZnO IN WATER-FREE ZINC SALT	ROTATION OF ZINC SALT
	Determination A	Determination B	Average		
	per cent	*per cent*	*per cent*	*per cent*	
1	13.05	12.97	13.01	33.18	*l*
2	13.68	13.47	13.575	33.18	*l*
3	13.49	13.41	13.45	33.15	*l*
4	12.90	13.00	12.95	33.33	*l*
5	13.43	13.56	13.495	33.57	*l*
Theoretical for optically active zinc lactate......			12.88	33.46	

The percentages of ZnO in the zinc salts agree with the theoretical value for zinc lactate, and the percentages of water of crystallization indicate that the lactic acid was optically active; the rotation of the zinc salt was *l* showing that the rotation of the free acid was *d*. The organism evidently produced lactic acid of the *d* type.

The volatile acid in the spoiled evaporated milk. The volatile acid present in the spoiled evaporated milk was investigated by the preparation and study of barium salts according to the methods used by Hammer and Sherwood (1923). The results obtained are given in table 6; samples 1 and 2 were from milk which coagulated at the condensery while sample 3 was from milk which coagulated following inoculation.

The percentages of barium in the barium salts suggest a mixture of acetic and propionic acids while the Duclaux values indicate largely acetic acid.

TABLE 6

Data on volatile acid in the spoiled evaporated milk

SAMPLE NUMBER	Ba IN BARIUM SALT			RESULT OF DUCLAUX DETERMINATION
	Determination A	Determination B	Average	
	per cent	*per cent*	*per cent*	
1	50.24	50.08	50.16	Largely acetic acid
2	50.01		50.01	Largely acetic acid
3	50.90		50.90	Largely acetic acid
Theoretical for Ba acetate.....			53.78	
Theoretical for Ba propionate..			48.46	

Proteolysis by B. coagulans. Although evaporated milk coagulated by *B. coagulans* does not show any evidence of being proteolyzed, members of the genus Bacillus often bring about protein decomposition in milk and, accordingly, a study was made of the soluble and amino-nitrogen contents of skim and evaporated milk coagulated by *B. coagulans*. A filtrate was secured from the milk by the method outlined by Hammer and Patil (1930); the soluble nitrogen was determined by the Kjeldahl method, and the amino nitrogen by the Van Slyke method. The results obtained are given in table 7.

The data show that in inoculated skim milk and in evaporated milk which had coagulated at the condensery there was an increase in both the soluble nitrogen and the amino nitrogen. In the skim milk the soluble nitrogen after growth of the organism was roughly twice that in the uninoculated milk while the amino nitrogen was roughly three times that in the uninoculated milk. In the evaporated milk which spoiled at the condensery the soluble nitrogen was almost twice that in the normal milk and the amino nitrogen more than twice that in the normal milk. The odor and flavor did not suggest putrefaction with either the skim milk or the evaporated milk.

TABLE 7

Proteolysis by B. coagulans

MATERIAL EXAMINED	SOLUBLE NITROGEN		AMINO NITROGEN	
	Per 10 ml. of filtrate	Increase over control	Per 10 ml. of filtrate	Increase over control
	mgm.	*mgm.*	*mgm.*	*mgm.*
Skim milk inoculated with:				
Culture 1	13.457	6.205	1.206	0.768
Culture 2	13.345	6.093	1.178	0.740
Culture 3	14.660	7.408	1.233	0.795
Culture 4	13.120	5.868	1.260	0.822
Culture 5	13.429	6.177	1.096	0.658
Culture 6	13.820	6.568	1.123	0.685
Uninoculated control	7.252		0.438	
Can of evaporated milk coagulated at the condensery	24.330	10.090	2.014	1.103
Can of normal evaporated milk from same lot	14.240		0.911	

Composition of gas from cans of spoiled evaporated milk. The composition of the gas from cans of spoiled evaporated milk was studied by collecting the gas over water and subjecting it to the usual absorption materials. The results secured on milk which coagulated following inoculation with *B. coagulans* and on milk which coagulated at the condensery are shown in table 8.

Oxygen was absent from the cans (except for the small amount in the control that had been opened and resealed) whether the milk had spoiled or was normal. This indicates that in normal milk the oxygen combines with the milk constituents. The coagulation of the milk was regularly accompanied by a large increase in the amount of carbon dioxide whether spoilage followed

TABLE 8

Gas content of cans of evaporated milk coagulated by B. coagulans

MATERIAL EXAMINED	TIME TO COAGULATE	PERIOD OF INCUBATION AT 37°C.	PERIOD OF HOLDING AT ROOM TEMPERATURE FOLLOWING INCUBATION	VOLUME OF GAS	OXYGEN		CARBON DIOXIDE		NITROGEN	
	days	*days*	*days*	*ml.*	*ml.*	*per cent of total gas*	*ml.*	*per cent of total gas*	*ml.*	*per cent of total gas*
Can of evaporated milk inoculated with:										
Culture 1	4	30	45	35.0			4.9	14.0	30.1	86.0
Culture 2	3	30	45	41.1			6.4	15.6	34.5	84.4
Culture 3	4	30	45	41.6			5.8	13.9	35.8	86.1
Culture 4	4	30	45	43.4			7.0	16.1	36.4	83.9
Unopened control		30	45	23.0			0.3	0.1	22.9	99.9
Opened and resealed control		30	45	34.1	0.4	0.1	0.5	0.1	33.2	99.8
Cans of evaporated milk which coagulated at the condensery:										
A			180	46.4			7.2	15.5	39.2	84.5
B			180	47.0			7.4	15.7	39.6	84.3
C			180	40.6			3.4	8.4	37.2	91.6
Cans of normal evaporated milk from same lot as cans A, B and C:										
D			180	37.6			0.6	0.2	37.0	99.8
E			180	41.4			0.6	0.2	40.8	99.8
F			180	41.0			0.6	0.2	40.4	99.8

inoculation or occurred naturally. In the spoiled cans carbon dioxide generally made up about 15 per cent of the gas. In one can which had spoiled at the condensery, approximately half the usual amount of carbon dioxide was found; the total acidity of the milk from this can was also definitely lower than usual.

It should be emphasized that *B. coagulans* does not produce a bulging of cans of evaporated milk in which it grows.

Influence of temperature on the rate of coagulation of evaporated milk inoculated with B. coagulans. Tables 2, 4 and 8 present data on the time required at 37° and at 50°C. for the coagulation of cans of evaporated milk inoculated with *B. coagulans*. Thirty-four cans held at 37°C. coagulated in from three to ten days (average 6.7 days) while six cans held at 50°C. coagulated in from three to four days (average 3.7 days). Six inoculated cans of milk held at 21°C., and two cans held at about 7°C. failed to coagulate during a period of four months. These results show the great influence of temperature on the growth of *B. coagulans*; they suggest that wide variations are to be expected in the time required for spoilage of milk containing *B. coagulans*, and that milk containing this organism may fail to show spoilage if the holding temperature is comparatively low.

SUMMARY

B. coagulans was isolated from an outbreak of coagulated evaporated milk that was similar to the one which yielded the original culture of this organism. The description of the organism was checked and enlarged.

Rather large numbers of organisms were found in coagulated evaporated milk that had been stored for varying periods at different temperatures. The great viability of the organism makes its isolation from such milk comparatively easy.

The total and volatile acidities of coagulated evaporated milk were much higher than those of normal evaporated milk. The non-volatile acid was *d* lactic acid while the volatile acid was apparently made up of acetic and propionic acids.

Although evaporated milk coagulated by *B. coagulans* does not show any evidence of proteolysis, this organism greatly increased both the soluble and amino nitrogen in skim milk and in evaporated milk. The coagulated milk did not have an odor or flavor suggesting putrefaction.

No free oxygen was found in the gas from cans of normal or coagulated evaporated milk. One can, which was opened and then resealed without being inoculated, was found to contain a small amount of oxygen. The carbon dioxide content of the gas from cans of coagulated milk was found to be about 15 per cent while that of normal milk was much lower.

Temperature had a marked influence on the rate of coagulation of evaporated milk inoculated with *B. coagulans*.

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COMPARATIVE STUDIES ON THE NATURAL AND ACQUIRED RESISTANCE OF CERTAIN STRAINS OF ESCHERICHIA COLI TO THE BACTERIOSTATIC AND GERMICIDAL EFFECTS OF CATIONS[1]

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HISTORICAL

The literature is replete with instances demonstrating that an increase in the resistance of a strain of microörganisms to a toxic substance is an adaptation which is frequently attained when successive generations of that strain are subjected to the influence of sublethal or subinhibitory quantities of the toxic substance.

Workers in the early days of specific chemotherapy were lead to postulate the development of drug-fastness in the organisms under study. This conception was first clearly formulated by Mesnil and Nicolle (1906). Ehrlich (1907a, 1907b, 1907c, and 1907d) and his co-workers and Browning (1907, 1908) were the first to demonstrate that the drug-fast character in trypanosomes may be retained in serial animal passages. Uhlenhuth, Hübener and Woithe (1907) were unable to demonstrate this transmissibility in durine infections treated with atoxyl. Mesnil and Brimont (1908a) and Plimmer and Bateman (1908) met with like failure in the treatment of experimental nagana with tartar emetic.

The transmissibility of the drug-fast character *in vivo* is, nevertheless, a general rule. Plimmer and Thomson (1907) encountered it in the treatment of experimental surra and nagana with atoxyl. Mesnil and Brimont (1908b) demonstrated it in a strain of *Trypanosoma evansi* resistant to atoxyl. A further complication of the subject is that the species of experimental animal used plays a large part in the inheritance of the drug-fast character. This has been clearly demonstrated by Breinl and Nierenstein (1908), Moore, Nierenstein and Todd (1908) and Mesnil and Brimont (1908c).

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[1] Contribution from the Department of Bacteriology of the University of Kentucky.

In vivo studies on organisms other than protozoa were first reported by Morgenroth and Kauffmann (1912) who observed the development of resistance to optochin in mice by pneumococci.

A distinct parallelism between resistance *in vivo* and that *in vitro* has been reported by Mesnil and Brimont (1908b) in studies on the use of tartar emetic in experimental surra. Broden and Rodhain (1908), however, in the treatment of human trypanosomiasis noted 1 case in which the resistance of a strain of *Trypanosoma lewisi* to tartar emetic was very great *in vitro* but was not apparent *in vivo*. Ehrlich (1909) noted the exact opposite in 1 case of experimental trypanosomiasis in the mouse. The organisms were highly resistant to partially oxidized arsenophenylglycine *in vivo* but were subnormally resistant *in vitro.* Gonder (1911) found that arsenic-fast strains of *Trypanosoma lewisi* obtained *in vivo* did not lose their resistant character on artificial culture media. The relation between *in vivo* and *in vitro* resistance has been found to be of great theoretical and experimental value by Feirer, Meader and Leonard (1926).

The demonstration *in vitro* of the power of adaptation in bacteria to the unfavorable environment produced by continuous cultivation in a medium containing antiseptics was first made by Kossiakoff (1887). Abbott (1891, 1912) observed an increase in the resistance of *Staphylococcus aureus* to the germicidal action of $HgCl_2$ by a process of artificial selection. Trambusti (1892) was able to produce an acclimatization to $HgCl_2$ in several species of organisms. Danysz (1900) produced an arsenic-fast strain of *B. anthracis.* Marks (1910) increased the resistance of a strain of *Salmonella suipestifer* to arsenious acid eight-fold and noted a simultaneous increase in its resistance to antimony of forty-fold. Altmann and Rauth (1910) reported the development of strains of *E. coli* which were markedly resistant to phenol. Masson (1910) found that the acquired resistance of his organisms to such substances as $HgCl_2$ $CuSO_4$, resorcinol and salicylic acid was temporary and might at any time be superseded by increased sensitivity.

Regenstein (1912) reported the development of resistance to cresol, saponified solution of cresol and to zinc sulphocarbolate in a strain of *Staphylococcus aureus* which had been grown in a medium containing phenol for several generations. He produced a strain resistant to $HgCl_2$ and found it to be resistant also to $HgBr_2$ and $Hg(CN)_2$. Meader and Feirer (1926) working with $AgNO_3$, mercurochrome, formaldehyde and acriflavin found that the drug-fastness obtained in strains of *E. coli*, *Eberthella typhi* and *Aerobacter aerogenes* was highly specific and that its development to one of these substances was often accompanied by an increased sensitivity to the others.

Beginning with the work of Dreser (1893) many workers have demonstrated that the dissociated portion of mineral acids and simple salts thereof is chiefly or perhaps entirely responsible for the effects of these substances on bacterial viability. He found that the inhibiting effect of mercuric salts on yeast could be counteracted by the addition of potassium hyposulphite. He explained this by the fact that the double salt formed does not give free mercuric ions on dissociation, a point well established by the behavior of its solutions in a potential gradient. This idea was in complete accord with the results of Koch (1881), Behring (1890), Heider (1892) and others who showed the direct relationship between rise in temperature and increase in disinfectant power.

The idea first formulated by Dreser has been confirmed and extended by many workers in the field of microbiology. Notable among these are the studies of Paul and Krönig (1896), Krönig and Paul (1897), Scheurlen and Spiro (1897), Bial (1897, 1902), Minervini (1898), Winslow and Lochridge (1906) and Chick (1908, 1910). Clark (1899) working with fungi was forced to disagree in part with this theory but his discordant results can be partially explained by the fact that he was compelled by the resistant nature of the organisms studied to use solutions which may not strictly be termed dilute. The exact boundary between dilute and concentrated solutions is a debatable point, however, and care should be taken not to define a solution of a mineral acid or a simple salt as concentrated merely because its effects on metabolic processes do not follow the laws of dilute solutions as based on physico-chemical principles.

Of numerous studies on the effect of cations upon bacterial growth those of Eisenberg (1918), Holland (1922) and Hotchkiss (1923) were the first to call attention to the fact that the various cations differ only in degree of potency. They have demonstrated that Na^+ and K^+, commonly known as favorable to bacterial growth, are markedly toxic in sufficiently high concentrations. Furthermore, these observers have noted that Pb^{++} and Hg^{++}, usually referred to as toxic, are stimulating in sufficiently high dilution, a fact previously noted for the effects of phenol, trikresol, $HgCl_2$ and arsenicals upon the growth of spirochaetes by Bronfenbrenner and Noguchi (1913). In this connection Shaughnessy and Winslow (1927) have shown that, whereas weak solutions of sodium salts increase the permeability of the bacterial cell wall, stronger solutions decrease its permeability. They have also shown this to be true for solutions of calcium salts although a considerable difference in degree of potency was noted. Moreover, Winslow and Dolloff (1928) have presented results which indicate that the cations in a solution containing a mixture of salts of univalent and bivalent metals exert an additive, not an antagonistic, effect upon bacterial viability. Their analysis of their results is inexact but the technical error involved is not great enough to impair the value of their findings seriously. This matter will be discussed in a later section. More recently Winslow and Haywood (1931) have extended these observations with essentially similar results.

OBJECT OF INVESTIGATION

The present studies were planned to determine in some measure the answers to the following questions:

1. Do the relative germicidal potencies of various cations follow each other in the same ascending order as the relative bacteriostatic potencies for each strain of the organism under observation when determined under identical conditions?

2. Does the development of increased resistance to one or more cations affect in any manner the degree of resistance of the same organism to other cations?

EXPERIMENTAL

Since it was desirable to compare the results of this investigation with the work of previous investigators in this field, *Escherichia coli* was chosen as the test organism. Two strains were used. Strain A was isolated from a contaminated water supply. Strain B, kindly furnished by Dr. C.-E. A. Winslow of Yale University, was the strain used by Hotchkiss (1923), by Winslow and Dolloff (1928), by Winslow and Haywood (1931) and by others working in the same laboratory.

All salts used were chlorides of the highest purity obtainable. All glassware used in these studies was carefully cleaned by placing it in dichromate cleaning solution for twenty-four hours with a subsequent series of four rinsings in warm distilled water.

The following media were used in determining the critical bacteriostatic concentrations of the salts:

1. The peptone solution, used by Hotchkiss (1923) and by Winslow and Dolloff (1928), consisting of 1 per cent Difco bactopeptone in distilled water.

2. Dolloff's synthetic medium, used by Winslow and Dolloff (1928), consisting of 5 grams Pfanstiehl c. p. lactose, 5 grams recrystallized ammonium tartrate and 0.02 gram dibasic ammonium phosphate in 1,000 cc. distilled water.

The critical bacteriostatic concentrations of the salts were determined upon a test suspension of the organism consisting of a twenty-four hour culture in either of the above media which had been incubated at 37°C. Inoculations of 0.05 cc. were made into tubes containing 5 cc. homologous medium and varying concentrations of the salt in question. These subcultures were incubated at 37°C. for forty-eight hours. The tubes were then examined for signs of growth. The *critical bacteriostatic concentration* was taken as the lowest concentration expressed as molality of the cation which showed complete inhibition of growth. The tube containing the highest concentration of salt showing growth was in each case streaked on Endo's medium for confirmation. All determinations were made in triplicate.

The germicidal efficiency of the various salts was determined at 37°C. The test suspension of the organism was prepared by centrifugalizing a twenty-four hour culture of the organism in Dolloff's medium and washing the sedimented bacterial cells three times by resuspending them in sterile distilled water with subsequent centrifugalization. The sedimented organisms were then suspended in sterile distilled water and the turbidity adjusted to match that of a standard suspension of $BaSO_4$ arbitrarily chosen and roughly approximating the density of an eighteen-hour culture of *E. coli* in Dolloff's medium. The bacterial suspension was warmed to 37°C. and used immediately. The salt solutions to be tested were made up in varying concentrations under aseptic conditions and 2.5 cc. of each concentration were placed in sterile test tubes provided with a cotton plug. These solutions were then warmed to 37°C. An inoculum of 0.25 cc. of the test suspension was placed in each tube and the whole shaken and replaced in the water bath at 37°C. An exposure of six hours was found to be the most satisfactory from the standpoint of greatest accuracy over the range of concentrations used. Subcultures were made by transferring one loopful of the mixture into double strength nutrient broth containing 0.5 per cent lactose. A platinum loop 4 mm. in diameter was used. These subcultures were then incubated at 37°C. for forty-eight hours and examined for signs of growth. All growth suspected of being due to contamination was streaked on Endo's medium for confirmation. The lowest concentration expressed as molality of the cation which would kill the test organism under the experimental conditions was regarded as the *critical germicidal concentration* for the time of exposure. The results were all checked by two or more separate determinations.

The resistance of each strain to two cations, Mg^{++} and Hg^{++}, was then increased by growing the organism in successive transplants in Dolloff's medium containing the highest concentration of the salt in question which would permit growth. From time to time this concentration could be increased until an apparent maximum was reached. The resistance of strain A to Mg^{++} was increased four-fold after 84 serial transfers; to Hg^{++}, thirty-fold after 79 serial transfers. The resistance of strain B to Mg^{++} increased three-fold after 90 transfers; to Hg^{++}, twenty-fold after 85 transfers.

TABLE 1

The critical bacteriostatic concentrations for strain A

CATION	MENSTRUUM	MOLALITY OF SALT
K^+	Dolloff's medium	0.75
	Peptone medium	1.00
Na^+	Dolloff's medium	0.70
	Peptone medium	1.00
Mg^{++}	Dolloff's medium	0.18
	Peptone medium	0.25
Ba^{++}	Peptone medium	0.18
Ca^{++}	Peptone medium	0.16
Fe^{+++}	Dolloff's medium	0.00025
	Peptone medium	0.001
Cu^{++}	Dolloff's medium	0.0005
	Peptone medium	0.0006
Pb^{++}	Peptone medium	0.0004
Hg^{++}	Dolloff's medium	0.00000025
	Peptone medium	0.00000075

These resistant substrains were then tested as before for the bacteriostatic and germicidal effects of the various cations upon them. The test suspensions in this case, however, were prepared from a twenty-four-hour culture in Dolloff's medium containing that concentration of the salt to which the organism's resistance had been enhanced.

The results obtained are recorded in tables 1 to 6. Table 2 contains the data of Winslow and Dolloff (1928) and of Hotchkiss (1923) upon the same organism.

TABLE 2

Critical bacteriostatic concentrations for strain B

CATION	OBSERVER	MENSTRUUM	MOLALITY OF SALT
K^+	Borman	Dolloff's medium	0.80
	Winslow and Dolloff	Dolloff's medium	0.80
	Borman	Peptone medium	0.95
	Winslow and Dolloff	Peptone medium	1.00
	Hotchkiss	Peptone medium	2.00
Na^+	Borman	Dolloff's medium	0.70
	Winslow and Dolloff	Dolloff's medium	0.60
	Borman	Peptone medium	0.90
	Hotchkiss	Peptone medium	2.00
Mg^{++}	Borman	Dolloff's medium	0.20
	Winslow and Dolloff	Dolloff's medium	0.20
	Borman	Peptone medium	0.30
	Hotchkiss	Peptone medium	0.50
Ba^{++}	Borman	Peptone medium	0.20
	Hotchkiss	Peptone medium	0.25
	Winslow and Dolloff	Asparagin medium	0.20
Ca^{++}	Borman	Peptone medium	0.18
	Winslow and Dolloff	Peptone medium	0.20
	Hotchkiss	Peptone medium	0.50
Fe^{+++}	Borman	Dolloff's medium	0.0005
	Borman	Peptone medium	0.0005
	Hotchkiss	Peptone medium	0.001
Cu^{++}	Borman	Dolloff's medium	0.00075
	Borman	Peptone medium	0.001
	Hotchkiss	Peptone medium	0.001
Pb^{++}	Borman	Peptone medium	0.0005
	Hotchkiss	Peptone medium	0.0005
	Winslow and Dolloff	Asparagin medium	0.0005
Hg^{++}	Borman	Dolloff's medium	0.0000005
	Borman	Peptone medium	0.000001
	Winslow and Dolloff	Peptone medium	0.0000005
	Hotchkiss	Peptone medium	0.000001

THEORETICAL DISCUSSION

The medium described by Dolloff (1926) furnishes an admirable menstruum for the study of salt action. Its relatively simple composition assures uniform results with several lots of media. This uniformity is a potent factor in an investigation so consuming of time and media. This medium affords a heavy growth of *E. coli* and, incidentally, of *Aerobacter aerogenes* which shows no tendency to become less dense in continuous serial transfers. Its main disadvantage lies in the fact that it is chemically incompatible with certain salts, such as $CaCl_2$.

TABLE 3

Critical bacteriostatic concentrations for resistant substrains of strain A in Dolloff's medium

(Concentration expressed as molality of salts)

CATION	STRAIN A	RESISTANT SUBSTRAIN	
		Mg^{++}	Hg^{++}
K^+	0.75	1.50	2.10
Na^+	0.70	1.50	2.00
Mg^{++}	0.18	0.72	0.90
Fe^{+++}	0.00025	0.0005	0.001
Cu^{++}	0.0005	0.001	0.005
Hg^{++}	0.00000025	0.0000005	0.0000075

TABLE 4

Critical bacteriostatic concentrations for resistant substrains of strain B in Dolloff's medium

(Concentration expressed as molality of salts)

CATION	STRAIN B	RESISTANT SUBSTRAINS	
		Mg^{++}	Hg^{++}
K^+	0.80	1.25	2.20
Na^+	0.70	1.25	2.00
Mg^{++}	0.20	0.60	0.80
Fe^{+++}	0.0005	0.00075	0.00125
Cu^{++}	0.00075	0.002	0.003
Hg^{++}	0.0000005	0.000005	0.00001

The practice adopted herein of expressing cation concentration in terms of molality is not technically correct and has no real quantitative significance. This practice has been adhered to, however, because of the lack of a better one. Winslow and Dolloff (1928) have adopted the use of the ionic activity. Their use of this term, however, has been very loose. Since the activity coefficient of an individual ion is dependent upon the total ionic strength of the solution, it would become necessary to know the exact concentration and valence of each ion in the menstruum

TABLE 5

Critical germicidal concentrations (six hours' exposure) for strain A and its resistant substrains

(Concentration expressed as molality of salt)

CATION	STRAIN A	RESISTANT SUBSTRAINS	
		Mg^{++}	Hg^{++}
Na^+	4.00	4.50	>6.15*
Mg^{++}	1.30	2.00	2.30
Ba^{++}	1.10	>1.6*	>1.6*
Ca^{++}	1.25	2.10	2.50
Fe^{+++}	0.0002	0.001	0.008
Cu^{++}	0.002	0.008	0.0125
Hg^{++}	0.00000005	0.0000002	0.0000005

* Saturated solution does not kill.

TABLE 6

Critical germicidal concentrations (six hours, exposure) for strain B and its resistant substrains

(Concentration expressed as molality of salt)

CATION	STRAIN B	RESISTANT SUBSTRAINS	
		Mg^{++}	Hg^{++}
Na^+	4.20	4.50	>6.15*
Mg^{++}	1.75	2.80	3.20
Ba^{++}	1.20	>1.6*	>1.6*
Ca^{++}	1.75	2.70	3.20
Fe^{+++}	0.0005	0.001	0.01
Cu^{++}	0.0025	0.01	0.025
Hg^{++}	0.0000001	0.0000008	0.000002

* Saturated solution does not kill.

employed. This is not always readily ascertainable. The calculation of the ionic strength in Dolloff's medium is complicated by the fact that certain cations form complexes with the ammonium ion and by the partial conversion of the carbohydrate to acid by the heat of sterilization. Finally, the inadequacy of the data available for the determination of ionic activities renders the use of this term impracticable for application to biological studies over more than a limited range of concentrations.

In this connection it is pertinent to note that Winslow and Dolloff (1928) have apparently confused molality with ionic strength. Whereas these two terms are equivalent for solutions of salts containing two univalent ions, this is not true of the solutions of any salt containing a bivalent ion. Reference to the Lewis and Randall (1923) tables shows the values for the activity of Mg^{++} as computed by Winslow and Dolloff to be erroneously derived. The magnitude of this error is not, however, great enough to render their conclusions unacceptable.

ANALYSIS OF TABULATED DATA

Before attempting to correlate the results obtained it is fitting to call attention to the remarkable agreement of the results of three investigations on the critical bacteriostatic concentrations for strain B. The results of this investigation are, with a few exceptions, in remarkable accord with the results of Hotchkiss and of Winslow and Dolloff. Hotchkiss's high values are partially explained by her use of a peptone medium which in our hands yielded values somewhat higher than those obtained with Dolloff's medium.

An analysis of the relative bacteriostatic potencies of the cations shows that for each of the strains of *E. coli* studied they can be arranged in the following ascending order: K^+ and Na^+; Ca^{++}, Mg^{++} and Ba^{++}; Cu^{++}, Fe^{+++} and Pb^{++}; Hg^{++}. This confirms the findings of Hotchkiss (1923) and of Eisenberg (1918) with a slight relative variation in the placing of Cu^{++}, Fe^{+++} and Pb^{++}.

The relative germicidal potencies follow in the same order for those so tested. *An acquired increase in the resistance to the* bacteriostatic action of Mg^{++} and of Hg^{++} induced comparable increases in this resistance to the other cations with each strain. This increased resistance was accompanied by a parallel rise in resistance to the germicidal action of all the cations tested. This non-specific stimulation of the mechanism of salt-resistance is not necessarily in disagreement with the results of Meader and Feirer (1926). It may be regarded as a group specificity which would not have been detected in such a diversified series of antiseptics as were employed by these authors. Attention is again called to the results of Marks (1910) who, by increasing the resistance of *Salmonella suipestifer* to arsenic, increased by an even greater quantity its resistance to antimony.

GENERAL CONCLUSIONS

Under the experimental conditions involved in so far as they apply to the specific cations and organisms studied the following conclusions are valid:

1. The relative bacteriostatic and germicidal potencies of cations follow each other in the same ascending series for each strain of the organism.
2. The germicidal potency of any cation occupies the same relative position in a series of such potencies as does the bacteriostatic potency in its homologous series.
3. Acquired resistance to the bacteriostatic action of one cation induces a comparable increase in resistance to the bacteriostatic and germicidal action of other cations with different chemical properties.

These considerations suggest that both the bacteriostatic and the germicidal effects of cations are produced by identical or intimately related properties, probably physico-chemical in nature. Furthermore, they indicate that the defense mechanism invoked by the organism undergoing artificial sitmulation of resistance to bacteriostatic and germicidal power is the same for all cations.

Assuming the theory of disinfection postulated by Bancroft and Richter (1931) to be true, these studies indicate that all cations function in the same manner in producing precipitation of the emulsoid colloids of the bacterial cell. It is possible that the newer knowledge of the effects of cations upon bacteria may form a working basis for the development of a general law covering the effects of salts upon emulsoid sols in general.

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AN IMPROVED METHOD FOR THE CULTIVATION OF ANAEROBIC MICROÖRGANISMS

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In 1915 Laidlaw demonstrated that heated platinized charcoal acted as a strong catalyzing agent and brought about the combination of hydrogen and oxygen to form water. One year later McIntosh and Fildes (1916) applied this principle and also suggested the use of palladiumized asbestos as a better catalyzer for the production of practical anaerobiosis for the cultivation of obligatory anaerobic microörganisms. Their apparatus was modified by Smillie (1917) and by J. H. Brown (1921, 1922). Boëz (1927) concluded, after a large experience with these jars, that further improvements from the point of view of safety, rapidity, prevention of leakage and multiple use of the same catalyzer, should be made. He suggested the use of a central catalyzing station which was electrically heated by a coil of nichrome wire attached to the outside of the catalyzing chamber.

In our experience complete anaerobiosis was more readily obtained and a more luxuriant growth of the obligatory anaerobes resulted when the catalyzing unit was placed within the culture jar. In order to eliminate all danger of explosions and still allow the deoxygenation of the jar to take place rapidly, we made use of an electric bulb as the heating unit for the palladiumized asbestos. This apparatus proved so satisfactory and was so free from the usual disadvantages and dangers associated with other similar methods that we feel a brief description of the apparatus is worthy of being placed on record.

APPARATUS

The apparatus consists of (*a*) the catalyzing unit, (*b*) the cylindrical museum jar, (*c*) the supply of electric current and (*d*) the source of hydrogen.

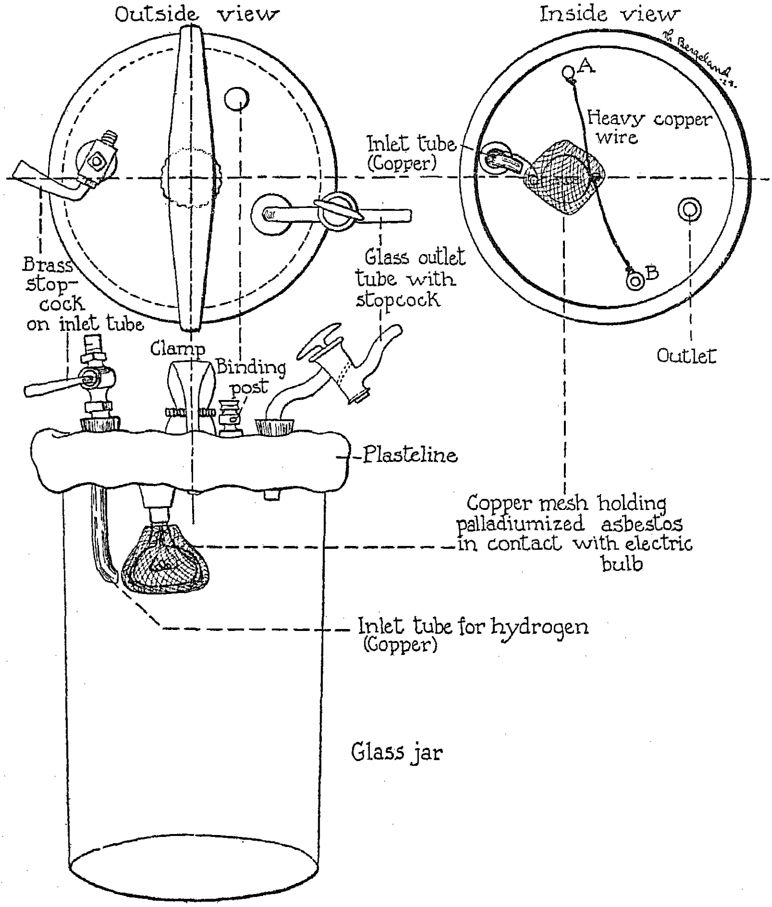
Outside view
Inside view
Th. Bergeland '29.
A
Heavy copper wire
Inlet tube (Copper)
B
Brass stop-cock on inlet tube
Glass outlet tube with stopcock
Outlet
Clamp
Binding post
Plasteline
Copper mesh holding palladiumized asbestos in contact with electric bulb
Inlet tube for hydrogen (Copper)
Glass jar

FIG. 1. A DIAGRAMMATIC PICTURE SHOWING IN DETAIL THE CONSTRUCTION OF THE SPECIAL LID AND THE HEATING UNIT FOR THE PALLADIUMIZED ASBESTOS

(*a*) The catalyzing unit is permanently attached to the under surface of the special brass lid of the culture jar (fig. 1). The lid is machined from a brass plate $\frac{3}{8}$ inch in thickness and properly fitted to the culture jar with which it is to be used. On the under surface of the lid a porcelain socket for the electric bulb is held in place by a heavy copper wire which is tightly stretched between the small bolt *A* and the binding post *B* in the diagram. Electrically, this connects one side of the lamp socket with the entire brass lid. The lamp circuit is completed by connecting the other side of the socket with the copper inlet pipe which extends through a rubber stopper in one of the large holes in the brass lid. A layer of palladiumized asbestos about $\frac{1}{4}$ inch in thickness is held in contact with the electric bulb by a fine copper mesh. It is important that the copper mesh completely surround the electric bulb and that no palladiumized asbestos extend outside the mesh. The inlet tube for hydrogen should be directed toward the copper mesh containing the palladiumized asbestos.

(*b*) The culture jars which we employ are the regular cylindrical museum jars about 7 inches in diameter and about 15 inches tall. The jars must have a flange at the top in order that the special lid can be held firmly in place by a metal clamp. The size of the jar is of little importance except for the fact that very large jars make it necessary to continue the process of deoxygenation a great deal longer.

(*c*) The electric bulb that has been found most suitable for this work is a 6-volt, 12-candle-power, carbon filament bulb of the screw-base type. Any current supply of 6 volts can be used. We employed a fixed rheostat that was made to deliver 6 volts and one and one-half amperes when used with the regular house current of 110 volts.

(*d*) Any type of hydrogen generator may be used to supply the free hydrogen. Since we were interested in simplifying the apparatus as much as possible, we have been using hydrogen from the ordinary storage tanks. In these tanks the gas is compressed to a relatively high degree; consequently it is necessary to use a reducing valve to lower the pressure and to control the amount of hydrogen that is introduced into the anaerobic jar. Wash-bottles containing water should be connected between the hydrogen tank and the culture jar so that the rate of flow of hydrogen can be determined easily.

METHOD

The steps in the operation of deoxygenating the culture jar are as follows:

1. Place the inoculated culture media in the anaerobic culture jar.
2. Place the special lid on the jar and fix it in place by the heavy metal clamp.
3. Apply moulding clay to the junction between the brass lid and the glass jar.
4. Open both the stopcocks situated on the lid.
5. Connect the rubber tube from the hydrogen tank to the brass stopcock.
6. Flush the culture jar with hydrogen to remove as much of the air as possible, then close the glass stopcock.
7. Adjust the reducing valve on the hydrogen tank so that only a small amount of hydrogen under a very low pressure enters the culture jar.
8. Turn on the electric current so that the small electric bulb within the jar will heat the palladiumized asbestos and thus bring about the catalyzing action.
9. After about five minutes close the brass stopcock and disconnect the tube from the hydrogen tank.
10. The current to the heating unit of the catalyzer should continue for approximately fifteen minutes.
11. The wires are then disconnected and the culture jar is placed in an incubator heated to 37°C.

RESULTS

This method has proved to be a clean and rapid means of obtaining isolated colonies of obligatory anaerobic microörganisms of the most sensitive types upon the surface of ordinary laboratory culture media. Facultative anaerobes fail to develop in these culture jars; consequently the usual contaminating organisms can easily be separated from the strict anaerobes. The temperature within the jar, even in the vicinity of the catalyzing agent, never rises above 37.2°C. even though the temperature of the palladiumized asbestos rises as high as 250°C.

This method proved highly successful in the isolation of anaerobic microörganisms from experimentally produced chronic abscesses of the lung (Herrmann and Cutler (1928) Weidlein and Herrmann (1928)). We shall have an occasion later to discuss that part of the problem in detail.

CONCLUSIONS

We feel that the use of the electric bulb as a heating unit for the palladiumized asbestos in the manner herein described overcomes the disadvantages of other similar methods, and provides us with a simple, clean, safe and effective means of producing an oxygen-free atmosphere for the cultivation of obligatory anaerobic microörganisms.

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TWO NEW SPECIES OF BACTERIA CAUSING MUSTINESS IN EGGS

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The development of musty odors in eggs has occasionally been responsible for considerable losses. This defect cannot be detected in the process of candling and, even when preparing broken egg products, an occasional egg is missed until after it has been mixed into a batch which is thus rendered unsatisfactory for consumption.

Jenkins and Hendrickson (1918) described the musty odor as sometimes resembling that emanating from old fillers and flats while at other times it seemed more like that from sprouting potatoes, or spoiled hay or chaff. They found that the odor was not always eliminated by cooking and that the bacterial content was as a rule, very low. Of 45 such eggs examined, 42 contained less than 100 organisms per cubic centimeter. Occasionally musty eggs were encountered which had green whites.

Penington and Jenkins (1918) reported some observations on this phenomenon and pointed out that the defect seemed to occur among eggs held in damp surroundings.

Turner (1927) made smears and agar slant cultures from musty eggs. Gram-negative short rods were observed in the stained smears. The cultures were incubated at 37°C. for several days but no growth developed. However, when held at room temperature for twenty-four hours, growth appeared and a strong odor of must came from the agar slant. Pure cultures were isolated and mustiness was reproduced in eggs. Morphological, cultural and physiological characteristics of the organisms isolated were reported. Turner proposed the name *Achromobacter perolens* for the organism. In a private communication (1929) the same investigator stated that in the two and one-half years which had elapsed since the organism was isolated, it had progressively lost its odor-producing properties, so that now this important characteristic is almost entirely lost. He states, however, that the odor was extremely strong and penetrating when the organism was first isolated, so much so that it became a nuisance in the whole building in which he worked.

Spanswick (1930) isolated an organism from a musty egg which produced must on agar slants and in eggs.

Titman (1928) says that a single musty egg will spoil a huge batch of cookie or cake dough. Conner (1930) stated that when musty eggs are encountered while breaking eggs for freezing in China, the containers are "dumped" and the utensils are washed and sterilized before being used again.

EXPERIMENTAL

Musty eggs were occasionally encountered at a commercial egg breaking plant in Des Moines, Iowa. Eleven were examined for gas-forming organisms of the colon group, and the number of viable organisms developing on nutrient agar at 20 and 37°C. was also determined. From table 1 it is evident that mustiness is not always associated with high bacterial counts. In 4 samples the counts were extremely low and in 2 others, they were not what would be considered very high. In several instances, musty eggs had green whites. Organisms causing mustiness were isolated from 4 of 12 samples examined.

The egg material was plated on agar in the usual manner. The plates were smelled after different periods to determine whether must was present. From the musty plates colonies were fished and inoculated tubes again smelled to detect those which were musty. Repeated smelling of the musty tubes seemed to paralyze the olfactory nerves, but organisms producing must were isolated from 4 of the samples.

About 50 colonies were fished from the plates of egg XI, 2 being finally chosen because of the great abundance of must produced; 20 from the plates of egg X16, of which 5 were chosen; and 30 from the plates of X17. Almost every tube developed mustiness. The plates incubated at 37°C. did not show mustiness, but when removed and allowed to incubate at room temperature, mustiness developed in twenty-four hours. Of 20 colonies from the plates of egg X41, only 2 developed mustiness. The musty organisms were isolated after the egg material had been frozen twenty-three days, but not from the fresh material of egg X41. Twenty-five cultures showing must were isolated from 4 musty eggs. These cultures were purified by replating on nutrient agar, fished, and smelled.

TABLE 1

Bacterial examination of musty eggs

LABORATORY NUMBER	DATE (1929)	BACTERIA PER GRAM		GAS FROM LACTOSE BROTH*	GAS FORMER	REMARKS
		20°C., 5 days	37°C., 2 days			
X1†	3/29	130,000	170,000	0		Green white, musty
X2	3/29	1,000	1,000	0		Yolk from X1
X9	4/16	35,000	15,000	10,000	Aerogenes	Distinctly musty egg
X14	4/18	250	130	0		Distinctly musty egg
X16†	4/18	6,300	1,700	1,000	Colon group absent	Very musty white and yolk mixed
X17†	4/25	12,000,000	9,200,000	10	Coli	Very musty odor
X24	5/9	100,000,000	100,000,000	1,000	Aerogenes	Strong musty odor
X26	5/12	60,000,000	25,000,000	10	Aerogenes	Musty mixed egg
X27	5/12	55,000,000	63,000,000	1,000	Coli	Slightly musty
X28	5/12	16,200,000	6,500,000	10,000	Aerogenes	Slightly musty
X60	5/18	1,000	1,000	0		Slightly musty
X41†	6/5	12,800,000	7,400,000	10,000	Coli + aerogenes	Slightly musty; had been frozen twenty-three days

* Reciprocal of highest dilution giving gas in lactose broth.

† Organisms causing must isolated from these samples.

Pure cultures were inoculated into fresh eggs aseptically removed from their shells and transferred to sterile 250 cc. Erlenmeyer flasks. One loop from an agar slant was used as the inoculum. The eggs were incubated at room temperature. Mustiness developed within four days in every egg and pure cultures (as determined by microscopical examination) which produced mustiness were recovered from all. Some of the organisms gave the egg white a greenish tinge, others did not.

Cultural characteristics of the isolated organisms were observed on agar, gelatin, broth, potato, litmus milk, plain milk, tryptophane broth, nitrate broth, lead acetate agar and starch agar. The catalase and Voges-Proskauer reactions were also observed. The above media, except tryptophane broth and lead acetate agar, were made according to the formulae in the Manual of Methods for Pure Culture Study of Bacteria (1926). The other 2 were Difco products.

Tests for the production of indol were made after growing the organisms in tryptophane broth for various periods. Two methods were employed on duplicates. One set was tested by mixing equal parts of para-dimethyl-amino-benzaldehyde and a saturated solution of potassium persulphate, moistening the end of the cotton plug with the mixture and pushing it down to within an inch of the liquid, after which the tubes were placed in a steaming water bath for five to ten minutes. A positive result was indicated by the development of a pink coloration on the cotton plug. To each duplicate tube there was added 0.2 cc. of the following mixture: 75 parts amyl-alcohol, 25 parts concentrated hydrochloric acid and 5 parts (Eastman) para-dimethyl-amino-benzaldehyde. A red ring on the surface of the liquid showed the presence of indol.

Gelatin liquefaction was determined by growing the organisms in 14 per cent gelatin at 20°C. and observing for five days.

The production of acetyl-methyl-carbinol was determined by making a qualitative test on glucose peptone cultures and in Clark and Lubs medium. A 10 per cent KOH solution was added to an equal volume of a five-day old culture incubated at 25°C. The tubes were left exposed to the air and read at frequent intervals up to twenty-four hours. Tubes were considered as giving a positive Voges-Proskauer reaction if an eosin-pink coloration appeared.

The presence of catalase was determined by adding 1 or 2 drops of 3 per cent hydrogen peroxide to a twenty-four-hour-old culture growing in a Petri dish on agar. Effervescence indicated the presence of catalase.

Streaks were made over the surface of starch agar plates to determine diastase production. After forty-eight hours the plates were flooded with an iodine solution. A positive reaction was indicated by a clear zone of hydrolysis around the area of the growth.

Production of H_2S was determined by blackening of lead-acetate agar.

The organic acid media were of the following compositions: dipotassium phosphate, 0.1 per cent; magnesium sulphate, 0.05 per cent; organic acid, 0.2 per cent, adjusted to pH 6.0 with NH_4OH; 1.5 per cent agar added as a solidifying agent; 10 cc. of 0.5 per cent aqueous solution of China blue per liter of solution added as an indicator. Inoculations were made from an agar slant on the surface of the organic acid agar. Utilization of the organic acid was indicated by decolorization of the China blue and growth on the surface.

Fermentation studies were carried out on the following carbohydrates, glucosides and polyalcohols.

Hexoses: Glucose, mannose, levulose, galactose.
Disaccharides: Sucrose, maltose, lactose, trehalose.
Trisaccharides: Raffinose, melezitose.
Polysaccharides: Dextrin, glycogen, inulin, starch.
Pentoses: Xylose, arabinose, rhamnose.
Polyalcohols: Dulcitol, glycerol, mannitol, sorbitol, erythritol, pectin and xylan.

The media for these studies were composed of peptone, 5 grams; dipotassium phosphate, 1 gram; the respective sugars, 0.3 per cent; 1.0 per cent Andrade indicator per liter. The reaction was adjusted to pH 7.0 to 7.1. Inoculations were made from agar slants.

As a check the fermentations were repeated using China blue (10 cc. of a 0.5 per cent solution per liter) as an indicator. Acid-production was indicated by red coloration of the Andrade's indicator and blue coloration of the China blue. The tubes were incubated at 23 to 25°C. Readings were made on the fifth day.

In the production of acid from carbohydrates the fermentation was from the top down. At the end of one day, a thin layer of acid about 1 cm. thick could be observed on the top of the medium. By the end of forty-eight hours it was about 3 cm. thick and in four to five days, fermentation was complete, after which most of the tubes rapidly became neutral or alkaline.

After the fermentation tests were completed, one loop of each organism was again inoculated into eggs which had been aseptically removed from the shell and transferred to sterile wide mouthed flasks of about 250 cc. capacity. Mustiness was produced and pure cultures of the organisms were recovered. Types B and C turned the egg-white somewhat greenish; type A did not. The egg white did not appear decomposed but became more viscous.

TABLE 2

Differential characterictics of organisms causing mustiness in eggs

GROUP	CULTURE NUMBERS	SOURCE OF EGG SAMPLE	ARABINOSE	ERYTHRITOL	RHAMNOSE		SORBITOL	TREHALOSE	LACTOSE	GELATIN LIQUEFACTION	PEPTONIZED MILK	FLUORESCENE IN MILK	NO_3 REDUCED TO NO_2 AND GAS	GLUCOSE PHOSPHATE REMAINING ACID	LITMUS MILK ACID
					5 days	20 days									
A	1, 4, 52, 53	XI, X41	−	−	−	+	−	−	+	−	−	−	−	+	+
B	19, 22, 25, 26, 27, 45	16X, X17	+	+	+	−	+	+	−	+	+	+	+	−	−
C	31, 32, 33, 34, 36, 37, 38, 39, 40, 41, 42, 43, 44, 46, 47	X17	+	+	−	+	+	+	−	+	+	+	+	−	−

Acid not produced by any culture from aesculin, amygdalin, arabitol, dextrin, dulcitol, glycogen, inulin, maltose, melezitose, pectin, raffinose, salicin, starch, sucrose, xylan.

Acid produced by all cultures from glucose, galactose, levulose, mannose, moderate from glycerol, mannitol, xylose. Indol −, catalase +, capsules +, motility +, litmus reduced +, diastase −. Voges-Proskauer −. Acid production from top down +.

Utilization of organic acids: Good growth on citric and uric acid agar. No growth on acetic, oxalic, sulphanilic, tartaric, salicylic and formic acid agar.

Morphology: Gram-negative rods not producing spores. Short on agar, longer and stouter from broth. Filaments often observed.

Temperature relationships and oxygen: Aerobe: 44°C., no growth; 37°C., scant or no growth; 33°C., good growth; 25°C., best growth; 10°C., slow growth.

Three distinct groups of organisms causing mustiness in eggs were evident on the basis of cultural and physiological characteristics as shown in table 2.

DESCRIPTIONS OF SPECIES OF BACTERIA CAUSING MUSTINESS IN EGGS

Type A. Pseudomonas graveolens nov. sp.

Morphology:

Form: Nutrient agar (twenty-four hours, 23 to 25°C.), short rods with rounded ends, occurring singly and in pairs; filaments and capsules often seen in stained mounts.

Spores: Not found.

Motility: Actively motile in twelve- to twenty-four-hour broth or agar cultures at room temperature.

Staining: Gram-negative; stained readily; often unevenly.

Cultural characters:

Agar slants: Abundant, viscid, opaque, glistening and of a butyrous consistency, slightly raised; after two days at 23 to 25°C.; odor of must very evident.

Potato: Abundant tan colored growth after two days at 23 to 25°C.; darkened; odor of must very strong and penetrating.

Nutrient broth: Strong clouding; moderate viscid sediment, a thin oily pellicle produced in two days at 23 to 25°C.; musty.

Gelatin stab: No liquefaction in thirty days.

Agar colonies: Growth rapid (23 to 25°C.); circular; slightly raised, smooth surface, entire edge; amorphous, internal structure.

Physiological Characters:

Milk: Reduction and coagulation after two weeks with an acid ring on the surface.

Indol: Negative after seven days at 23 to 25°C. in tryptophane broth.

Nitrates: Not reduced to nitrites at 23 to 25°C. in seven days.

Hydrogen sulphide: Produced in two days at 23 to 25°C.

Oxygen: Aerobe.

Temperature: Growth absent at 43°C., scant at 33°C., abundant at 23 to 25°C., and slight at 10°C.

Diastatic action: Negative on starch agar in two days (23 to 25°C.).

Catalase: Produced in forty-eight hours at 23 to 25°C.

Acetyl-methyl-carbinol: Was not produced.

Glucose phosphate agar: Nutrient agar plus 0.5 per cent glucose and 0.1 per cent K_2HPO_4 and Andrade's indicator turned acid in two days at 23 to 25°C. and remained acid.

Fermentation: Neither acid nor gas was produced from the following in five days at 23 to 25°C.: aesculin, amygdalin, arabitol, dextrin, dulcitol, glycogen, inulin, maltose, melezitose, pectin, raffinose, salicin, sorbitol, starch, sucrose, xylan, arabinose, erythritol, or trehalose. Acid, but no gas, was produced from rhamnose, glucose, galactose, mannose, levulose, a moderate amount from xylose and a small amount from glycerol and mannitol. Growth occurred in agar containing lactic, uric and citric acids but not with acetic, oxalic, sulphanilic, tartaric, salicylic and formic acids as the sole sources of carbon, nitrogen being supplied as ammonia.

Type C. Pseudomonas mucidolens nov. sp.

Morphology:

Form: Nutrient agar (twenty-four-hours, 23 to 25°C.) longer rods than type A; rounded ends, occurring singly and in pairs; capsules occasionally seen in stained smears.

Spores: Not found.

Motility: Actively motile in twenty-four hour-broth or agar cultures.

Staining: Gram-negative; stained readily; filaments and granules frequently observed.

Cultural characters:

Agar slant: Abundant, viscid, opaque, glistening, spreading and of butyrous consistency, flat, transparent in two days at 23 to 25°C. odor of must very pronounced.

Potato: Abundant growth, pale olive in color; medium darkened; strong musty odor.

Nutrient broth: Heavy clouding; moderate viscid sediment; thin oily surface growth; musty.

Gelatin stab: Growth best at top; liquefaction (stratiform) beginning in two days; musty.

Agar colonies: Circular, slightly raised with a smooth surface; edge entire, internal structure amorphous.

Special agar: Nutrient agar with 0.1 per cent glucose, 0.1 per cent $MgSO_4$, 0.1 per cent K_2HPO_4 and 0.5 per cent NaCl was greened (fluorescent).

Physiological characters:

Milk: Slowly reduced and rapidly peptonized; curd was not observed (23 to 25°C.).

Indol: Negative after seven days at 23 to 25°C.

Nitrates: Nitrites and gas were produced in twenty-four hours. Nitrite was not present after two days (23 to 25°C.).

Hydrogen sulphide: Produced (slight).

Oxygen: Aerobe.

Temperature: Growth scant at 37°C., absent at 43°C., abundant at 23 to 25°C., and slight at 10°C.

Diastatic action: Negative.

Catalase: Produced.

Acetyl-methyl-carbinol: Not produced.

Glucose phosphate agar: Became acid in forty-eight hours (at 23 to 25°C.), after five days the reaction was neutral.

Fermentation: Neither acid nor gas was produced from the following carbohydrates: aesculin, amygadalin, arabitol, dextrin, dulcitol, glycogen, inulin, maltose, melezitose, pectin, raffinose, salicin, starch, sucrose, or xylan. Acid was produced from rhamnose in twenty days. Acid but no gas was produced from the following in five days at 23 to 25°C.: arabinose, erythritol, sorbitol, trehalose, glucose, galactose, levulose, mannose; moderate amount of acid was produced from glycerol, mannitol and xylose. Growth occurred on agar with lactic citric and uric acids, but not with acetic, oxalic, sulphanilic, tartaric, salicylic and formic acids as the sole sources of carbon, nitrogen being supplied as ammonia.

Type B. Pseudomonas mucidolens variety tarda nov. sp.

Type B is practically identical with type C in morphological and cultural characters. Physiologically it is slightly different, inasmuch as it produces acid from rhamnose in five days and then becomes alkaline. It requires two weeks for type C to produce acid from rhamnose. Type C utilizes lactic acid more quickly than does type B. Type B is considered as a variety of type C.

DIFFERENTIATION FROM PREVIOUSLY DESCRIBED ORGANISMS PRODUCING MUSTY ODOR

The organisms described above differ from Turner's organisms in the milk reaction. Type A coagulates milk with acid production, types B and C peptonize milk. Turner's organisms did not affect milk.

Type A does not liquefy gelatin or reduce nitrates. Type B and C do, as did Turner's organisms.

Type B and C produce fluorescence in agar when 0.1 per cent $MgSO_4$, 0.1 per cent glucose and 0.5 per cent sodium chloride are added. Turner's organism did not produce fluorescence; however, he states that the egg white from which the organism was isolated showed a greenish tinge.

Turner did not report the production of acid from erythritol, rhamnose, trehalose or sorbitol, the utilization of organic acids, the production of diastase, catalase, or acetyl-methyl-carbinol. Turner's organism corresponds very closely to types B and C but differs from them in the failure to produce any change in milk. Spanswick's organism did not grow at 37°C., liquefy gelatin, reduce nitrate, or produce any change in litmus milk. The fermentation characters were not reported. Spanswick's organism corresponds somewhat to type A, but differs from it in failure to coagulate milk.

SUMMARY

Eleven musty eggs were examined for bacterial content and attempts were made to isolate the organisms responsible for the musty odor. The causative organism was isolated from four.

Twenty-five must-producing cultures were studied as to morphological cultural and physiological characters. Two species and one variety are recognized for which the following names are suggested: *Pseudomonas graveolens, Pseudomonas mucidolens* and *Pseudomonas mucidolens* var. *tarda*. Each of these organisms produced a strong musty odor in agar, potato, gelatin, and broth media and when inoculated into eggs.

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MARINE BACTERIA COMMONLY FOUND ON FRESH FISH[1]

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Harrison (1929) has carried on a detailed investigation of various species of bacteria isolated from freshly-caught halibut and from sea-water at depths of 30 to 60 fathoms in the Pacific Ocean. A similar study of bacteria from the Atlantic was made at this laboratory and it is interesting to note definite similarities in the two groups. Comparing the Atlantic isolations with Harrison's list of organisms, the following species are found to occur in both groups:

Micrococcus varians Migula
Micrococcus citreus Migula
Micrococcus candidus Cohn
Rhodococcus agilis (Ali-Cohen) Holland
Flavobacterium turcosum (Zimmerman) Bergey
Flavobacterium fucatum Harrison
Achromobacter pellucidum Harrison

Two other species among the Pacific bacteria were isolated from cured fish at this laboratory. These were *Micrococcus aurantiacus* and *Sarcina lutea.* In addition, the Atlantic group contains organisms closely resembling *Micrococcus nitrificans* Bergey; *Micrococcus halophilus* Bergey; *Achromobacter geniculatum* (Wright) Bergey; *Achromobacter ambiguum* (Wright) Bergey; *Flavobacterium annulatum* (Wright) Bergey.

Achromobacter pellucidum and *Rhodococcus agilis,* both predominating forms, are of special interest. The repeated isolations of the former organism confirm in detail Harrison's original description (1929). This organism was isolated from halibut, haddock, and cod slimes (50 to 60 fathoms), also from sea-water. *Rhodococcus agilis* was obtained from the slimes of fresh cod and haddock, from smoked haddock, and ice fillet (flounder).

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Certain prominent marine forms are definitely proteolytic, and, as invaders or inhabitants of fish muscle, are of economic significance. *A. pellucidum,* one of this group, grew well at temperatures ranging from 9° to 30° in codfish gelatin. Growth occurred also at 3° but proteolytic activity was arrested at this temperature. *Pseudomonas fluorescens* exhibited a greater proteolytic action in codfish gelatin, though consideration of this organism in this connection requires explanation. *Ps. fluorescens* appeared among the Atlantic isolations from the surface of fresh halibut. This result is not in accord with Harrison's findings. He reports the absence of the Pseudomonas on fish freshly caught and states that sea-water from a depth of 30 fathoms contained no *Ps. fluorescens.* Refrigerator ice was shown to be an important source of the organism. In connection with the isolation reported here no ice had been previously employed; transfers were made directly from the body slime to sterile media as the fish were removed from the water. However, in view of the wide distribution of the organism and its presence in the holds of fishing vessels, on bin boards and landing nets, there is the possibility of contamination through brief contact with surfaces harboring this species. Whatever the source, the finding of *Ps. fluorescens* on the surface of fish freshly caught has a direct economic bearing upon the keeping quality of the product. To the list of proteolytic marine species, *Achromobacter geniculatum* and *Flavobacterium fucatum* should undoubtedly be added.

The formation of slimy or viscous growths is common in marine cultures. This property is often particularly pronounced in broth, gelatin, or on potato which has been previously immersed in salt solution. *A. pellucidum, A. viscidum* N. Sp., and *Ps. fluorescens* yield highly viscous cultures. *Achromobacter viscidum* was obtained repeatedly from sea-water and haddock slime. It resembles *A. litoralis* (Russell) Bergey, differing, however, in the arrangement of flagella. The former organism is monotrichous, using Casares-Gil's method.

Numerous species of halophilic bacteria were isolated. *Sarcina lutea, Sarcina subflava, Staphylococcus albus, Micrococcus candicans* and *Micrococcus aurantiacus* occurred prominently in pickled fish. The Sarcinae were also present in concentrated pickle brine and are probably marine. Of the sea-water isolations mentioned above, *A. ambiguum, M. nitrificans*, and *M. halophilus* proved to be salt-tolerant. Under the most favorable conditions for pickling, filamentous bacteria and torulae predominated. The fish in these cases were in good condition. In other instances where there were often evidences of decomposition, the cocci predominated. These species find conditions favorable for growth in salt concentrations as high as 10, 15, and 20 per cent.

Among the bacteria isolated from frozen fish were several marine species including *Micrococcus citreus, Rhodococcus agilis*, and *Micrococcus candidus*.

From the results presented it is reasonable to conclude that a more or less definite bacterial flora occurs on the surface of fresh fish and that there are marked similarities between the Atlantic and the Pacific isolations. It is interesting to observe that forms closely related to the above organisms are frequently encountered from other than marine sources. Cured fish frequently contain marine bacteria which are capable of pronounced proteolytic activity in fish muscle.

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AN IMPROVED REAGENT FOR THE ACETYL-METHYL-CARBINOL TEST

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It has become increasingly apparent that no single test is sufficient to separate the colon group from the aerogenes group of bacteria. Of the various tests in use the Voges-Proskauer test seems to be one of the most important. There is no doubt but that a number of strains of bacteria which do not belong to the aerogenes group also give a positive V.-P. test. In the writer's collections of some 260 strains of Gram-negative bacilli there are seven paratyphoid bacilli and three monoflagellated organisms which are V.-P. positive. None of the typical sucrose fermenting colon bacilli were V.-P. positive. In spite of much recent criticism of the test it seems to the author to be still one of the best for the differentiation of coli and aerogenes.

Because of the fact that the standard test requires so long for its completion various improvements have been suggested. For a review of the literature and also for a recent improvement the reader is referred to an article by Werkman (1931). For some reason or other the author has not found Werkman's technique to be much better than the standard technique. It has also the disadvantage of being more cumbersome than the standard technique, in that two separate reagents have to be added to the culture instead of one.

A new technique has been discovered which is just as simple as the standard technique and by which a positive test is obtained in from ten to twenty minutes. The new technique uses copper as a catalyst. The reagent is made as follows: One gram of copper sulphate ($CuSO_4 \cdot 5H_2O$) is dissolved in 40 cc. of concentrated ammonium hydroxide (specific gravity 0.90). This solution is added to 960 cc. of at least 10 per cent sodium hydroxide solution. (A solution of sodium hydroxide weaker than 10 per cent may lead to the precipitation of the copper.) The resulting blue solution is stable if kept in a stoppered bottle. As in the standard test, equal quantities of the reagent and of the culture are mixed. If the mixture is allowed to stand at room temperature, without shaking, a bright red coloration forms at the surface in from ten to twenty minutes. Immediately upon mixing the reagent and the culture a slight purplish coloration forms throughout the tube. This fades in a few minutes and must not be confused with the bright red coloration of the positive test which develops later at the surface, or throughout the tube upon shaking.

Salts of copper other than the sulphate may be used. If so, they must be added in equivalent concentration as determined by their molecular weights. If potassium hydroxide is used it should have a concentration of 15 per cent.

The copper reagent is somewhat more sensitive to acetyl-methyl-carbinol than the standard reagent. Some cultures which give a weak test with the standard reagent may be found definitely positive with the copper reagent. Also, some cultures which are negative with the standard reagent may be found weakly positive with the copper reagent. Whether this is an advantage or a disadvantage can only be determined by experience. The cultures used to test the new reagent were all incubated for five days in the standard medium before the test was made. No comparative studies were made at any other periods of incubation.

SUMMARY

The addition of 0.1 per cent copper sulphate to the sodium hydroxide of the Voges-Proskauer test renders the reagent more sensitive to acetyl-methyl-carbinol. The rate of the reaction is greatly increased so that a bright red coloration appears in from ten to twenty minutes.

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THE SELECTION OF A DILUTION WATER FOR BACTERIOLOGICAL EXAMINATIONS

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In making quantitative determinations of the number of bacteria in a sample which will grow on plates or in tubes, it is usually necessary to dilute a portion of the sample with sterile water to reduce the bacterial content to numbers which can be accurately enumerated. The dilution water may influence bacterial counts thus obtained in at least two ways: it may be bactericidal, and it may contain precipitates which will appear as confusing particles, simulating bacterial colonies on the plates, when the counts are made. Tests for the presence of such interference should be made on the water after it has been sterilized and is ready for use, as the characteristics of a water are frequently changed by sterilization.

Standard Methods for the Examination of Water and Sewage (American Public Health Association) in the first edition (1905) and in the second edition (1912) state that either sterile tap or distilled water may be used for dilution purposes in bacteriological work. In the third, and in all subsequent editions, sterile tap water is recommended and distilled water is not mentioned.

At first thought it would seem that a tap water would be ideal for bacteriological dilution. Upon careful investigation, however, only a few tap waters are found to be entirely suitable. Well, or spring, waters as a rule contain iron and a fairly large amount of mineral salts which are held in solution by carbon dioxide in the water. Precipitates are frequently formed when these waters are exposed to air, and are practically always produced by sterilization with heat. The hydrogen-ion concentration of such waters may be changed sufficiently by sterilization to make them bactericidal. Tap waters derived from surface sources are not constant in their composition. They not only vary in their natural mineral salt content but also from the effects of coagulation and chlorination.

In the actual field experience of this laboratory it has frequently been found necessary either to resort to waters other than tap water or to treat the tap water before it was used for bacteriological dilution because the available tap supply was not suitable after sterilization. For instance the hydrogen-ion concentration of Cincinnati, Ohio, tap water is ordinarily so low after autoclaving, pH 8.6 to 9.5, that it is bactericidal unless it is allowed to stand for at least forty-eight hours before use to give it time to reach an equilibrium with the carbon dioxide in the laboratory air. In actual practice it is allowed to stand for ninety-six hours before use. At times when the excess lime treatment is employed to avoid tastes and odors, the water as it comes from the tap is in the pH zone of 9.0 to 9.5 and greater difficulties are encountered.

TABLE 1

Hydrogen ion concentration of various synthetic dilution waters and natural waters before and after sterilization by autoclave

WATER OR SOURCE OF WATER	HYDROGEN ION CONCENTRATION EXPRESSED IN pH		
	Before sterilization	After autoclaving	Forty-eight hours after autoclaving
Distilled	6.7	8.5*	7.2
Bicarbonate	8.0	9.2	8.9
Phosphate	7.3	7.4	7.4
Formula C	7.3	7.4	7.4
Cincinnati, Ohio	8.1	8.6	7.6
Pine Ridge, S. D.	7.6	8.4	8.2
Lake Michigan	8.0	8.7	8.2
Charleston, S. C.	6.4	7.3	7.3
Lawrence, Kansas	7.8	8.2	8.1
Lynn, Mass.	7.0	8.5	8.2
Springfield, Ill.	7.9	8.6	8.2

* Distilled water changes to pH 7.2 to 7.4 within a short period after sterilization or immediately upon agitation.

Additional evidence indicating the changes in hydrogen-ion concentrations which are produced in waters when they are autoclaved is presented in table 1. In this table are given the pH values of the various natural and synthetic waters used in this study, as observed before autoclaving, after autoclaving and cooling, and forty-eight hours after autoclaving. In eight of the eleven waters examined changes in hydrogen-ion concentrations of 0.5 to 1.8 pH were observed.

Another factor that must be considered in the preparation of bacteriological dilution waters is the quality of the glassware in which the water is sterilized. Collins and Riffenburg (1925) have called attention to the dangers of pollution with materials dissolved from glass. Phosphate-buffered water, pH 7.2, autoclaved in pyrex glass containers, showed an unchanged final hydrogen-ion concentration. A portion of the same water after sterilization in a bottle of poor quality glass had a pH of 8.7. Poorly buffered waters undergo still greater changes. All dilution bottles used in this study were tested by filling with distilled water, autoclaving and then examining the water. The solution of material from glass is most rapid in distilled water. Bottles producing a persistent change in the hydrogen-ion concentration of the water were not used.

Similar difficulties have been encountered in selecting a dilution water for use in biochemical oxygen demand determinations. In this instance the criterion for the suitability of the dilution water is even more exacting, for not only must the water be favorable to the continuous viability of the biological factors concerned but it must also be free from any substance which would add to the oxygen requirement of the sample or interfere chemically with the quantitative determination of oxygen. Considerable progress has been made toward the development of a dilution water for this purpose. Mohlman, Edwards and Swope (1928) proposed a dilution water prepared by dissolving 0.5 gram of sodium bicarbonate per liter of distilled water. Later Mohlman (1930) has suggested that the bicarbonate content be reduced to 0.3 gram per liter. Theriault *et al.* (1931) have reviewed the results presented by these and other workers. In addition they made a comparative study of the oxygen demand results obtained when distilled water, bicarbonate water, and two synthetic waters (phosphate water[1] and formula C[1] water) prepared by them were used for dilution. As a result of this study and with a view to the eventual development of a dilution water for general use, they believe that it is desirable to standardize on the readily prepared phosphate dilution water.

As biochemical oxygen demand tests and bacteriological examinations are usually made in the same laboratory, it would be a decided step toward standardization and simplicity of operation if the same dilution water could be used for both procedures. With this object in view, a preliminary study on the selection of a suitable water has been made. In this study the results obtained with Cincinnati tap water, distilled water, bicarbonate water (300 p.p.m.), phosphate water, and formula C water have been compared to determine the relative suitability of these waters for bacteriological dilution. In each instance the source water was also used as a sixth dilution water. That is, as each sample was received for examination a portion of it was withdrawn, measured into dilution bottles, sterilized and used as a dilution water for that particular sample. All dilution waters except these source waters were sterilized at least forty-eight hours before they were used.

In making such a comparison it would be expected that some of the dilution waters might vary in their suitability depending on the nature and the source of the water sample under examination. In order to test this possibility and to obtain information adapted for more general application, samples for examination, using these dilution waters, were obtained from: (1) White Clay Creek, Pine Ridge Indian Agency, Pine Ridge, South Dakota, (2) Lake Michigan, near the mouth of the Chicago River at Chicago, Illinois, (3) Goose Creek, at Charleston, South Carolina, (4) Kansas River at Lawrence, Kansas, (5) Saugus River watershed at Lynn, Massachusetts, (6) Sangamon River at Springfield, Illinois, and (7) Ohio River at Cincinnati, Ohio. These are fairly representative of waters in the United States with the exception of the extremely alkaline waters of the far west.

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[1] The following stock solutions were used in the preparation of these dilution waters:

1. Phosphate buffer solution (34.0 grams KH_2PO_4 dissolved in 500 ml. of distilled water, adjusted to pH 7.2 with 1 M NaOH solution and made up to 1 liter with distilled water.
2. Calcium chloride solution, 0.10 M (18.3 grams $CaCl_2 \cdot 4H_2O$ per liter of distilled water).
3. Magnesium sulphate solution, 0.04 M (9.9 grams $MgSO_4 \cdot 7\ H_2O$ per liter of distilled water).
4. Ferric chloride solution, 0.001 M (0.27 grams $FeCl_3 \cdot 6\ H_2O$ per liter of distilled water).

Phosphate dilution water. The phosphate water was prepared by adding 1.25 ml. of stock phosphate buffer solution per liter of distilled water.

Formula C water. Formula C water was prepared by adding to each liter of phosphate dilution water stock solutions as follows: 0.1 M calcium chloride, 2.5 ml.; 0.04 M magnesium sulphate, 2.5 ml., and 0.001 M ferric chloride, 0.5 ml.

An attempt was made to determine the bacterical count, as observed in the various diluted samples: (1) immediately after the portion of the sample was added to the dilution water, (2) after it had been in the diluting water fifteen minutes, and (3) again after a thirty-minute exposure. Examinations were not made after longer periods because in routine procedure the sample would not ordinarily remain in the dilution water for a greater time than thirty minutes before the diluted sample would be mixed with the culture medium.

METHODS

In carrying out these tests the sample was vigorously shaken, and then 1 ml. was withdrawn and added to the first dilution water. The time was noted. The mixture was then shaken vigorously and 1-ml. portions were withdrawn and placed in Petri dishes. Four plates were made of each dilution and standardized pipettes were used. The plates were poured with agar at once and the time noted. The diluted sample was then allowed to stand for fifteen minutes before it was again vigorously shaken and portions withdrawn for plating. During this fifteen-minute period the process was repeated with the same sample for each of the dilution waters. The plants for the fifteen- and thirty-minute periods were made in the same manner. The interval during which the portion of the diluted sample stood in the Petri dish before agar was added did not exceed one minute. This avoided the adverse effects of sedimentation and evaporation which become factors if portions of a sample are allowed to stand in plates for fifteen minutes or over before the culture medium is added.

Three workers coöperated on each test. One acted as timekeeper, the second made the dilutions and added the 1-ml. portions to the Petri dishes, and the third looked after the agar and poured the plates.

The same agar was used throughout a given test. This agar was melted and cooled to 40°C. before the plating was started and was held at this temperature. Four plates were made of each dilution at each planting. Each count recorded in the tables represents the average of at least four plates from one dilution. The plates were cooled immediately after pouring and mixing, placed in a 37°C. incubator and incubated for twenty-four hours before the colonies were counted.

Although the exposure of the organisms in the sample to the dilution water, before the addition of agar, was accurately timed, the immediate planting period did not represent a zero time exposure to the diluting water, for some time was required to mix the portion of the sample with the water thoroughly and to add the required portions to the plates. As actually observed, this period of exposure was usually eighty seconds with an occasional variation of ten seconds.

A possible source of error that could not be fully controlled was that some change might take place in the number of organisms in the sample during the nine to ten-minute interval which elapsed while the portions of the sample were being added to the various dilution waters under test. While any change in such a short time would not ordinarily be expected, an attempt was made to balance it by varying the order in which the dilution waters were used in each test. No variations in the initial counts obtained were observed and it was concluded that the number of organisms in the sample did not change during this period.

RESULTS

The results secured in this comparative study of the bacterial counts obtained on samples from seven different sources, using six different dilution waters for each sample, are presented in tables 2 to 8.

For convenience in comparing the results, the actual immediate counts have been given in each instance and the subsequent counts obtained at the fifteen- and thirty-minute periods are expressed in percentages of the initial count. This was done in order that possible individual variations in the initial counts obtained with the different diluting waters might not influence the

TABLE 2

Results with sample from White Clay Creek, Pine Ridge Indian[1] *Agency, Pine Ridge, S. D.*

DILUTION WATERS USED	BACTERIA PER MILLILITER IN THE DILUTED SAMPLE AFTER FOLLOWING INTERVALS:			
	Immediate		Per cent of initial	
	Actual count	Per cent	15 minutes	30 minutes
Source water	208	100	96	108
Distilled	196	100	70	67
Bicarbonate	215	100	60	50
Phosphate	220	100	89	86
Formula C	206	100	105	113
Cincinnati tap	214	100	93	102

TABLE 3

Results with sample from Lake Michigan near mouth of Chicago River, Chicago, Ill.

DILUTION WATERS USED	BACTERIA PER MILLILITER IN THE DILUTED SAMPLE AFTER FOLLOWING INTERVALS:			
	Immediate		Per cent of initial	
	Actual count	Per cent	15 minutes	30 minutes
Source water	124	100	135	137
Distilled	133	100	86	59
Bicarbonate	117	100	80	51
Phosphate	137	100	86	84
Formula C	133	100	117	107
Cincinnati tap	129	100	103	95

TABLE 4

Results with sample from Goose Creek at Charleston, South Carolina

DILUTION WATERS USED	BACTERIA PER MILLILITER IN THE DILUTED SAMPLE AFTER FOLLOWING INTERVALS:			
	Immediate		Per cent of initial	
	Actual count	Per cent	15 minutes	30 minutes
Source water	152	100	97	98
Distilled	160	100	74	62
Bicarbonate	147	100	56	48
Phosphate	146	100	97	96
Formula C	149	100	95	97
Cincinnati tap	146	100	97	102

TABLE 5

Results with sample from Kansas River at Lawrence, Kansas

DILUTION WATERS USED	BACTERIA PER MILLILITER IN THE DILUTED SAMPLE AFTER FOLLOWING INTERVALS:			
	Immediate		Per cent of initial	
	Actual count	Per cent	15 minutes	30 minutes
Source water	151	100	87	81
Distilled	130	100	95	82
Bicarbonate	128	100	83	70
Phosphate	140	100	93	85
Formula C	143	100	108	84
Cincinnati tap	138	100	97	85

TABLE 6

Results with sample from Saugus River watershed at Lynn, Mass.

DILUTION WATERS USED	BACTERIA PER MILLILITER IN THE DILUTED SAMPLE AFTER FOLLOWING INTERVALS:			
	Immediate		Per cent of initial	
	Actual count	Per cent	15 minutes	30 minutes
Source water	380	100	99	105
Distilled	369	100	74	47
Bicarbonate	384	100	87	58
Phosphate	382	100	107	101
Formula C	392	100	101	104
Cincinnati tap	373	100	101	112

percentages. As a matter of fact only slight variations were observed in these initial counts, for the average initial counts of the seven samples obtained with each dilution water were: with Cincinnati tap water 175, phosphate water 179, formula C water 180, bicarbonate water 172, distilled water 173, and the source waters 179.

TABLE 7

Results with sample from Sangamon River at Springfield, Illinois

DILUTION WATERS USED	BACTERIA PER MILLILITER IN THE DILUTED SAMPLE AFTER FOLLOWING INTERVALS:			
	Immediate		Per cent of initial	
	Actual count	Per cent	15 minutes	30 minutes
Source water	213	100	105	106
Distilled	199	100	82	74
Bicarbonate	190	100	63	50
Phosphate	201	100	114	109
Formula C	211	100	108	106
Cincinnati tap	199	100	105	122

TABLE 8

Results with sample from Ohio River at Cincinnati, Ohio

DILUTION WATERS USED	BACTERIA PER MILLILITER IN THE DILUTED SAMPLE AFTER FOLLOWING INTERVALS:			
	Immediate		Per cent of initial	
	Actual count	Per cent	15 minutes	30 minutes
Source water	27	100	93	96
Distilled	27	100	63	41
Bicarbonate	24	100	67	54
Phosphate	30	100	97	87
Formula C	27	100	89	100
Cincinnati tap	27	100	93	96

Apparently the relatively short exposure for eighty seconds of the organisms in the sample to the dilution water, before the immediate planting was made, was not sufficient to affect the results materially in any instance. However, when the results for the fifteen- and the thirty-minute exposure periods are considered, it is observed that both the bicarbonate and the distilled water gave very low results for both periods with all samples. This general agreement in the results from all samples makes it appear inadvisable to use either distilled or bicarbonate waters for bacteriological dilution at any time. The results obtained from all the samples with the other four dilution waters were about as consistent among themselves as would be expected. The average results for all samples with each dilution water are presented in table 9.

Beyond a doubt the bactericidal influence of distilled water was due to the complete absence of mineral salts. Direct microscopic observations on living protozoon cells have shown that these cells are usually ruptured when they are placed in distilled water or in waters with a mineral salt concentration which varies widely from that of the medium in which the organisms are found. It is probable that many bacteria are similarly affected when they are placed in distilled water.

TABLE 9

Average results with samples from all sources with each dilution water

DILUTION WATERS USED	BACTERIA PER MILLILITER IN THE DILUTED SAMPLE AFTER FOLLOWING INTERVALS:			
	Immediate		Per cent of initial	
	Actual count	Per cent	15 minutes	30 minutes
Source water	179	100	102	105
Distilled	173	100	78	62
Bicarbonte	172	100	73	55
Phosphate	179	100	100	96
Formula C	180	100	104	103
Cincinnati tap	175	100	99	106

In the case of the bicarbonate water the bactericidal effect might be due to the low hydrogen ion concentration induced in part by heat sterilization, or the toxicity of the bicarbonate water as prepared. To test this point, bicarbonate water (300 p.p.m.) was prepared in three ways: (1) by sterilizing distilled water and, after cooling and shaking, adding the required amount of sodium bicarbonate with aseptic precautions, (2) by filtration of the bicarbonate solution through a Berkefeld W filter, and (3) by the usual procedure of autoclave sterilization. The hydrogen ion concentrations, expressed in pH for the three solutions thus prepared, were 8.1, 8.3, and 9.1, respectively. Using these three bicarbonate waters with sterilized Cincinnati tap water as a control dilution water, samples of Ohio River water were examined. The results obtained are given in table 10.

These results indicate quite clearly that the bactericidal action of the bicarbonate dilution water was due to the low hydrogen-ion concentration and not to the toxicity of the bicarbonate per se.

TABLE 10

Results with sample from Ohio River at Cincinnati, Ohio, using bicarbonate dilution waters sterilized by various means

DILUTION WATERS USED	BACTERIA PER MILLILITER IN THE DILUTED SAMPLE AFTER FOLLOWING INTERVALS:			
	Immediate		Per cent of initial	
	Actual count	Per cent	15 minutes	30 minutes
Bicarbonate, A*	48	100	67	62
Bicarbonate, B*	48	100	110	100
Bicarbonate, C*	49	100	98	90
Cincinnati tap	50	100	102	102

* The three bicarbonate waters used were sterilized as follows: (A) by autoclaving in regular manner, (B) by sterilizing distilled water and preparing solution with aseptic precautions, and (C) by filtering through a Berkefeld W filter.

Control plates of all three waters were sterile.

DISCUSSION

Two prerequisites of synthetic dilution waters for bacteriological use are indicated by the results obtained; they involve the mineral salt content, and the hydrogen-ion concentration. The results with distilled water indicate that the presence of some mineral salt is imperative. Of the mineral salts employed in this study the amount, providing the concentration is within the range found in natural waters, and providing unfavorable hydrogen-ion concentrations are not produced, does not appear to affect the results. Hydrogen-ion concentrations as low as pH 8.2 were used without reducing bacterial numbers during a thirty-minute period. However, at pH 9.0 a very marked decrease in bacterial numbers was observed after fifteen minutes' exposure. This indicates that when mixed cultures of bacteria are being considered, many of the bacteria are killed or at least rendered inactive at a pH between 8.2 and 9.0.

At this point in the consideration of dilution water effects, distinction must be made between the death of the bacterial cell (as evidenced by an inability to grow when transferred from a dilution water to a suitable culture medium), and the ability to grow without lag in a medium diluted with such a water. Butterfield (1929) and Parsons *et al.* (1929) have shown that certain bacteria in dilute media grow best, and without lag, at a pH of about 7.0. Undoubtedly with mixed cultures of bacteria, as the hydrogen ion concentration changes from the zone of pH 7.0, where optimum growth is obtained, to the zone of pH 9.0, where bactericidal effects are observed, varying conditions of growth will be encountered. At the lower pH range the majority of the bacteria will grow well and without lag. In the intermediate zone growth will occur but with increasing periods of lag. Finally in the higher pH range many of the bacteria will fail to grow altogether or may even be destroyed.

The evidence presented by Theriault *et al.* (1931, pages 1099–1100) indicates that when distilled or bicarbonate dilution waters are used for biochemical oxygen demand determinations there is a decided lag in the oxidation rate for at least the first day of incubation. This lag was most marked in the higher dilutions and the effect persisted longer with distilled water. In the same article (pages 1112–1113) additional data are presented to show the influence of variations in seeding on biochemical oxygen demand results, particularly marked during the first day of incubation and occasionally persisting to the end of a test. The data presented at this time, which show that in distilled and in autoclaved bicarbonate dilution water 40 to 50 per cent of the bacterial flora, added in polluted water, are rendered inactive in thirty minutes or less, offer a very probable explanation of the low oxygen demand results obtained when distilled water is used. In the case where unsterilized bicarbonate dilution water is used the hydrogen ion concentration of the mixture, although not low enough to be bactericidal, is in the zone where a definite lag in the bacterial growth of mixed cultures is induced. Many of the bacteria present in sewage, and probably many protozoa also, find conditions in such mixtures unsuitable for growth; they pass out of the field of action and a lag of several hours intervenes before the residual organisms, which are able to survive and to grow, multiply in sufficient numbers to produce normal oxidation. Under such conditions, not only is a lag produced by the limited activity during the first day of incubation but the biological flora and fauna acting may also be limited to a lesser number of species and the effect produced by this limitation would persist.

SUMMARY

In this study, in which the results obtained in the bacteriological examination of samples from seven widely separated locations in the United States are compared, using five different dilution waters which have been suggested, the following summary appears to be warranted.

1. Phosphate dilution water and formula C water give the most consistent results.

2. With a view to the development of a dilution water which can be used for both bacteriological and oxygen demand tests, it seems desirable to standardize on the readily prepared phosphate water for further study as it forms the basis for the more complete formula C water.

3. In the bacteriological examination of natural waters the dilution water employed must contain some mineral salts. The amount of mineral salts required in the diluting water, within the range found in natural waters, does not appear to be critical so far as the survival of the bacteria is concerned. If growth of the bacteria, without lag, is desired, it is probable that a degree of mineralization corresponding to that of the natural water would be more favorable.

4. The hydrogen-ion concentration of the dilution water also does not appear to require critical adjustment, providing it is not lower than pH 8.2, if survival of the bacteria for a short period

only is desired. However, if growth of the bacteria without lag is desired, a pH of 7.5 should probably not be exceeded.

5. Distilled water and dilution waters with hydrogen-ion concentrations in the zone of pH 9.0 or lower are decidedly bactericidal.

6. In making tests on the suitability of dilution waters, the examinations should be made after the water has been sterilized, for the sterilization process may very greatly alter the characteristics of the water. This is particularly true for tap and bicarbonate waters.

7. Consideration must also be given to the glass container in which the dilution water is sterilized. Material dissolved from glass bottles of poor quality during autoclaving may make marked changes in the reaction and in the mineral salt content of the water.

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OXIDATION-REDUCTION POTENTIALS IN RELATION TO THE GROWTH OF AN AEROBIC FORM OF BACTERIA[1]

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During the past few years a great many studies have been made on the electromotive properties of biological systems. In general, the studies indicate that bacterial cultures as well as sterile bacteriological media are active oxidation-reduction systems. The effect produced by the bacteria on the potentials of the substratum on the one hand, and the effect of the potentials of the substratum on the growth of the organisms on the other, involve relations of very definite significance.

In a previous communication, Allyn and Baldwin (1930) reported that marked effects on the growth of an aerobic form of bacteria were induced by certain changes in the oxidation-reduction character of the media. In continuation of these studies an endeavor has been made to study by electrometric measurements the oxidation-reduction potentials developed in certain bacteriological substrata and to interpret the same experimentally in terms of bacterial growth. Certain irregularities were encountered in these studies with aerobic organisms, rhizobia; yet some significant correlations, it is believed, were manifested between the potential-time curves of the substrata and the ability of the organisms to initiate growth therein.

EXPERIMENTAL

Methods

A simple potentiometer circuit was found unsatisfactory in making potential measurements in these poorly poised biological systems because of the polarization effects produced by a current of the magnitude characteristic of this circuit, passing through the system when finding balance. The polarizing effects were avoided by the use of a vacuum-tube potentiometer designed in this laboratory.[2] This circuit was found later to differ only in certain details from that used by Plotz and Geloso (1930) in their studies.

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[1] Published with the permission of the Director of the Agricultural Experiment Station.

A diagram illustrating the principal features of the vacuum-tube potentiometer is given in figure 1. In the circuit shown in this diagram, when the switch *S* is thrown to the right, the plate current has a definite value, determined by the magnitude of the negative grid bias *C*. This current causes a deflection of the galvanometer in the plate circuit. By giving the resistance R_1 a suitable value, this current may be balanced out so that there is no deflection of the galvanometer.

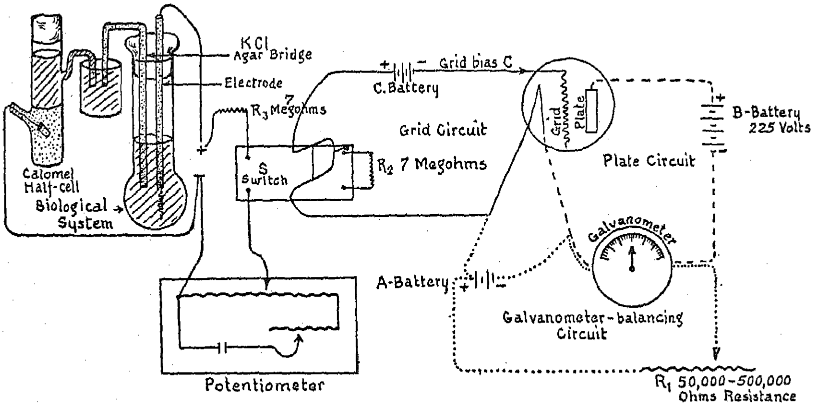

FIG. 1. DIAGRAM OF THE VACUUM-TUBE POTENTIOMETER

Now when the switch *S* is thrown to the left, the E.M.F. to be measured becomes a part of the negative grid bias *C*. The plate current then is changed and again there is a deflection of the galvanometer. The potentiometer is connected also in the grid circuit so that any potential generated by it is a part of the negative grid bias. If the potentiometer be adjusted so that the potential generated by it is equal and opposite to the potential to be measured, the net result is that the negative grid bias becomes the same as before and the galvanometer once more reads zero.

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[2] Credit is due Mr. M. Johnson for the design of this circuit.

In brief then, the E.M.F. to be measured is brought into the system by throwing the switch S to the left. If the potentiometer be adjusted until the galvanometer returns to zero, it is evident that the reading on the potentiometer will be equal to the potential being measured.

The initial negative grid bias C is so adjusted that the grid current has a very low value (approximately 10^{-10} amperes). Such a current was found to have a negligible polarizing effect on the electrodes in the systems investigated. No drifting of the galvanometer obtained within the time necessary to complete the readings, and the readings were consistently reproducible.

The saturated calomel half-cell was used as a standard of comparison in actual operations. By interpolation from the tables of Clark (1928) the saturated calomel half-cell has been calculated to have a value of $E_h = 0.243$ volt at 28°C., the approximate temperature at which all the readings were made in these experiments. The data submitted have been corrected to the standard normal hydrogen electrode, $E_h = 0$, by simply adding 0.243 volt to the observed E.M.F.

The electrode vessels used in these studies were large test tubes (1 by 8 inches) blown out somewhat larger at the base in order to prevent the electrodes from coming in direct contact with the glass during operations (fig. 1). These tubes were of uniform size so that 50 cc. rendered the depths of the substrata in the different experiments comparable to those commonly employed in test-tube culture work. Two electrodes were inserted into each tube and in all cases at approximately the same level (2 inches) beneath the surface of the medium.

The electrodes consisted of bright platinum wire sealed in the end of small soft glass tubing, circuit contact being made by means of mercury. The difficulty arising from the cracking of electrodes during cleaning and sterilization was almost completely avoided by the use of 22 gauge platinum wire and by flowing only a small amount of glass into the seal about the platinum. The electrodes were cleaned in chromic-sulphuric acid, rinsed thoroughly in distilled water, and flamed in an alcoholic flame before each experiment.

The electrodes, together with the tube for the KCl-agar bridge, were suspended firmly by a cotton plug in Pyrex test tubes (1 by 8 inches) and autoclaved at 15 pounds for twenty minutes. Subsequently the KCl-agar bridge was filled aseptically and the cotton plug with the electrodes and KCl-agar bridge, were bodily set over into a tube of the same diameter containing the system to be studied. After sterilization, the tubes holding the platinum electrodes were filled with mercury by means of a capillary pipette.

Some discrepancies were noted in the initial potential readings of comparable systems depending upon the method employed in the sterilization of the electrodes. Electrodes autoclaved while immersed in distilled water frequently indicated potentials approximately 50 millivolts more positive than similar set-ups in which the electrodes were autoclaved while merely suspended by cotton plugs in the empty Pyrex tubes. The electrodes sterilized under water were as a rule in agreement with each other and the same was true of those sterilized in steam, indicating rather definitely that the method of sterilization was responsible for the apparent differences in the observed readings.

These differences in potential readings were of greatest concern in attempting to make potential measurements on sterile bacteriological media. The electrodes sterilized by the two methods were brought into good agreement when oxidizing or reducing compounds, such as hydrogen peroxide or thioglycollic acid respectively, were introduced into the system, augmenting its electromotively active properties. In inoculated media, the electrodes sterilized by the two methods came into good agreement as soon as the bacterial growth was well under way.

OXIDATION-REDUCTION PROPERTIES OF STERILE YEAST-WATER MANNITOL AND MANNITOL NITRATE LIQUID MEDIA

Two basic media were employed in these studies. The constituents of the two media were as follows:

1. Yeast-water mannitol medium

Distilled water	900 cc.
Yeast water*	100 cc.
Mannitol	10.0 grams
$MgSO_4 \cdot 7H_2O$	0.2 gram
K_2HPO_4	0.5 gram
NaCl	0.1 gram

2. Mannitol nitrate medium

Distilled water	1,000 cc.
KNO_3	1.0 gram
Mannitol	10.0 grams
$MgSO_4 \cdot 7H_2O$	0.2 gram
K_2HPO_4	0.5 gram
NaCl	0.1 gram

* The yeast water was prepared by autoclaving sterile, fresh, starch-free bakers' yeast in 10 times its weight of tap water, allowing to settle and decanting the clear supernatant liquid.

The media were prepared fresh and adjusted to pH 7.0 for each experiment. The freshly autoclaved media in tubes containing 50 cc. quantities were cooled rapidly to approximately 37°C. and the electrodes together with the KCl-agar bridge inserted at once. Care was taken not to shake the tubes during the operations. Some of the tubes of media were covered immediately with a $1\frac{1}{2}$-inch vaseline seal. The systems were then placed in a 28°C. incubator preparatory to making the potential measurements.

The potential-time curves of the two media with vaseline seals, under the conditions just described, are depicted in figure 2. It should be understood that the observations recorded here can represent only in a very general way the oxidation-reduction properties of these two sterile media. As previously pointed out the methods of sterilizing the electrode altered the initial readings of such systems. The results reported here were obtained with electrodes sterilized under distilled water. Electrodes autoclaved in steam gave readings more negative by approximately 50 millivolts. This difference seemed to persist in the aerobic tubes, but in the media under vaseline seals the difference between electrodes sterilized by the two methods gradually disappeared as the potentials of the system dropped. Moreover, in the case of the yeast-water mannitol medium, different batches of the yeast-water used and even portions of the same batch taken at different ages seemed to influence the observed potentials of this medium.

In a general way, however, both of the freshly autoclaved media under aerobic conditions showed a positive drift of potential for the first twelve to eighteen hours, during which time the media were approaching equilibrium with atmospheric oxygen. Thereafter the curves flattened, with a subsequent very slow positive drift over a period of several days. The mannitol-nitrate medium, under these conditions followed a course some 30 to 50 millivolts more positive than the yeast-water mannitol medium.

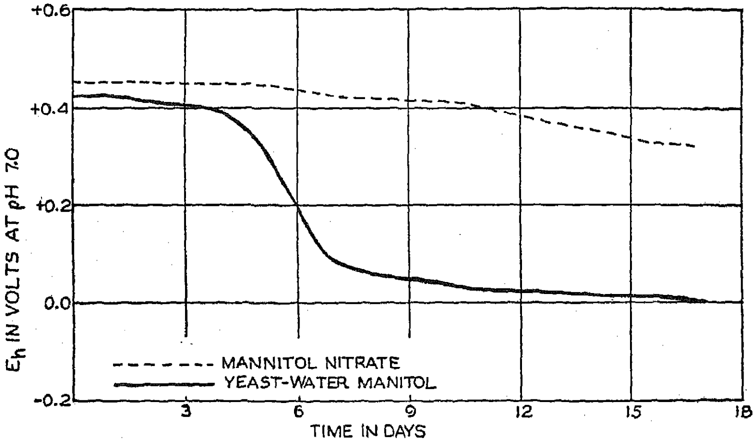

Fig. 2. Potential-time Curves of Sterile Yeast-water Mannitol and Mannitol Nitrate Media under Vaseline Seals

A very noticeable difference was observed, however, in the electromotively active properties of these two media when placed under a vaseline seal. It is recognized that vaseline seals may not be wholly impervious to atmospheric oxygen, yet it is probable that diffusion through a $1\frac{1}{2}$-inch seal is very slight during the time alloted to the experiments. At any rate the conditions were comparable for the two media under investigation.

The mannitol-nitrate medium exhibited a negative potential drift of only about 12.5 millivolts over a period of seventeen days, whereas the yeast-water mannitol showed a negative potential change of some 425 millivolts (fig. 2). The striking contrast, in the reducing character of these two media when placed under vaseline seals is probably related to the difference in the abilities of the two media to support the growth of *Rh. trifolii.*

MEDIUM	DILUTIONS OF THE ORGANISMS, RH. TRIFOLII						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Yeast-water mannitol	+	+	+	+	+	+	+
Mannitol nitrate	−	−	−	−	−	−	−
Mannitol nitrate, plus 0.015 per cent $CaCl_2$	+	+	−	−	−	−	−

The yeast-water mannitol medium permits growth of *Rh. trifolii* (clover nodule organism, 202) in high dilutions, a 10^{-7} dilution of a five-day culture; whereas a 10^{-1} dilution of the organisms seldom manifests growth in the mannitol-nitrate medium.

Some evidence of improvement was obtained by changing the ion-balance of the mannitol-nitrate medium with the addition of 0.15 gram $CaCl_2$ per liter. In this case, the clover nodule organism was able to initiate growth in a 10^{-2} dilution, but only after a lag phase of some fourteen to seventeen days. It will be seen in data to follow, that when the potential of this mannitol-nitrate medium is properly reduced, growth in much higher dilutions results even without the addition of the $CaCl_2$; but greater improvement in growth is procured when both the $CaCl_2$ and a reducing agent are added.

OXIDATION-REDUCTION PROPERTIES OF CERTAIN MODIFICATIONS OF THE MANNITOL-NITRATE MEDIUM IN RELATION TO BACTERIAL GROWTH

It has just been indicated that the mannitol-nitrate medium under a vaseline seal showed only a very slight negative potential drift as contrasted with the yeast-water mannitol medium. Further, the nitrate medium did not support growth of *Rh. trifolii* except when large inocula were used and the nutritive balance improved by the addition of $CaCl_2$. Even then growth occurred only after an extended lag phase.

Data presented in a previous communication by Allyn and Baldwin (1930) showed that growth was obtained with high dilutions of the organisms after the addition of cysteine or powdered agar to this nitrate medium. The cysteine introduced another factor, an organic nitrogen source, aside from a change in the oxidation-reduction potentials of the system. In the present studies, thioglycollic acid, a nitrogen-free reducing -SH compound, has been substituted for the cysteine. The potential-time curves of the various modifications of this substratum have been followed by electrometric measurements, and the effect of such potentials on the growth of the organisms in comparable systems determined.

Effect of thioglycollic acid and of agar. The effects of various concentrations of thioglycollic acid and of powdered agar on the growth of rhizobia in the nitrate medium without $CaCl_2$ are shown in the first part of table 1.

The medium was prepared fresh, and tubed in 8 cc. quantities. The thioglycollic acid, approximately 13 N, was found to be sterile. It was adjusted aseptically to pH 7.0 with sterile N/14 NaOH before being added to the medium.

The powdered agar was dried and autoclaved in a petri plate. From 0.2 to 0.4 gram was added to 10 cc. of medium after seeding the organisms.

A six-day culture of the organisms in a yeast-water mannitol medium was used as the source of the inocula for the various dilutions. It is recognized that some of the ingredients of the yeast-water medium were carried over with the inocula. As this procedure was uniformly followed, however, the conditions were comparable for all the experiments.

In these experiments none of the dilutions of *Rh. trifolii* was able to initiate growth in the control tubes containing the untreated mannitol-nitrate medium. With the addition of thio-

TABLE 1

The effect of thioglycollic acid and of powdered agar on the growth of Rh. trifolii 202 in the mannitol nitrate medium with and without the addition of $CaCl_2$

MEDIUM WITH NO $CaCl_2$	DILUTIONS OF ORGANISMS						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Check	–	–	–	–	–	–	–
Check +0.002 per cent thioglycollic acid	+?	–	–	–	–	–	–
Check +0.005 per cent thioglycollic acid	+++	–	–	–	–	–	–
Check +0.0075 per cent thioglycollic acid	+++	+++	++	+	+	+	–
Check +0.01 per cent thioglycollic acid	+++	+++	++	+	+	–	–
Check +0.025 per cent thioglycollic acid	+++	+++	+	–	–	–	–
Check +0.05 per cent thioglycollic acid	+++	++	–	–	–	–	–
Check + powdered agar 4 per cent	+++	+++	+++	+++	+++	+++	–
MEDIUM PLUS 0.015 PER CENT $CaCl_2$							
Check	+++	+++	–	–	–	–	–
Check +0.002 per cent thioglycollic acid	+++	+++	–	–	–	–	–
Check +0.005 per cent thioglycollic acid	+++	+++	+++	–	–	–	–
Check +0.0075 per cent thioglycollic acid	+++	+++	+++	+++	+++	+++	+++
Check +0.01 per cent thioglycollic acid	+++	+++	+++	+++	+++	+++	+++
Check +0.025 per cent thioglycollic acid	+++	+++	+++	–	–	–	–
Check +0.05 per cent thioglycollic acid	+++	+++	–	–	–	–	–
Yeast-water mannitol medium	+++	+++	+++	+++	+++	+++	+++

1. Readings recorded after seventeen days' incubation.
2. Inocula taken from six-day culture in yeast-extract mannitol medium.
3. +'s = amount of growth as judged by turbidity.

glycollic acid up to and including 0.0075 per cent, growth conditions were gradually improved as the amounts of the reducing compounds were increased. Progressive increases in the concentrations of the thioglycollic acid above 0.0075 per cent, produced a corresponding decrease in the number of dilutions showing growth.

Even though growth occurred in dilutions as high as 10^{-6} when the optimum amount of thioglycollic acid was added to the nitrate medium, much heavier growth occurred in the tubes receiving the larger inocula, as shown by the turbidity of the cultures. This would indicate that certain ingredients of the yeast-water medium carried over with the large inocula rendered the nitrate medium capable of supporting a larger growth of the organisms. Such nutrients alone, however, were ineffective in establishing suitable conditions in the nitrate medium for growth initiation, since no growth occurred in the control tubes to which no thioglycollic acid had been added (table 1).

The fact that heavy growth was obtained throughout in the tubes receiving the sterile powdered agar, lends some evidence to support the belief that certain inclusion materials of the commercial agar actually improved the nutritive balance of the medium. Other effects of the agar will be discussed presently.

Thioglycollic acid plus $CaCl_2$. In the first part of this paper it was pointed out that the mannitol-nitrate medium supported growth of the large inocula of rhizobia (10^{-1} and 10^{-2}) when 0.015 per cent $CaCl_2$ had been added to the medium; whereas under otherwise comparable conditions no growth occurred. This led to another experiment in which both the $CaCl_2$ and various amounts of thioglycollic acid (0.002 to 0.05 per cent) were added to the tubes of nitrate medium. The calcium chloride was sterilized separately in solution and added in proper concentrations to the tubes of sterile medium at room temperature to prevent the precipitation of the phosphates.

Under these conditions, the 10^{-1} and 10^{-2} dilutions of the organism were able to initiate growth in the tubes of nitrate medium to which the $CaCl_2$ but no reducing agent had been added. Visible signs of clouding did not appear with the 10^{-2} dilution however until after thirteen to fifteen days' incubation. The 10^{-3} dilution rarely showed visible signs of growth even after a period of one month (see table 1).

When the optimum amount of thioglycollic acid (0.0075 per cent) was added to this nitrate medium together with the $CaCl_2$, heavy growth obtained uniformly up to and including the 10^{-7} dilution of the organisms. Growth here was as good as that obtained in the yeast-water mannitol medium (table 1).

Electrode potentials of comparable systems. An attempt was made to follow, in a general way, the potential-time curves developed in the sterile media under the conditions just described. The procedure followed in making the electrometric determinations was as described in the discussion of methods.

In spite of certain irregularities encountered while working with media under aerobic conditions, definite correlations were manifested between the potentials of the substrate and the ability of the organisms to initiate growth under the conditions set forth in table 1. The fact that 0.002 per cent thioglycollic acid failed to render the nitrate medium favorable for growth, while, on the other hand, 0.0075 per cent made growth possible with high dilutions of the organisms may be explained, it is believed, in terms of the difference in potential levels established under these two conditions.

The 0.002 per cent thioglycollic acid produced a sudden, but temporary, fall in the potential of the medium, followed by a rapid rise within twenty-four hours back to some 374 millivolts positive to the normal hydrogen electrode. The curve then straightened with slow positive drift over a period of several days (fig. 3).

The addition of 0.0075 per cent thioglycollic acid (the optimum amount) caused a somewhat similar but lower dip in potential of the system followed by a much more gradual rise over a period of three to four days. After the fourth day the curve began to flatten but at a potential some 50 millivolts negative to the former (0.002 per cent). The lower region of potential maintained by the 0.0075 per cent thioglycollic acid would appear to account for the ability of the smaller inocula to initiate growth in this case and not in the other (fig. 3).

Conversely, the addition of 0.05 per cent thioglycollic acid obviously created conditions too reduced for optimum growth.

A pronounced lag phase was observed with the large inocula (10^{-1} and 10^{-2} dilutions) and complete inhibition with the smaller inocula. This amount of thioglycollic acid carried the potential of the medium to a very low level, 160 millivolts negative to the normal hydrogen electrode, and maintained the potential in this region for several days. Only a very gradual positive drift of the system was observed over a period of eight days due to the capacity factor characteristic of this concentration of the reducing agent (fig. 3).

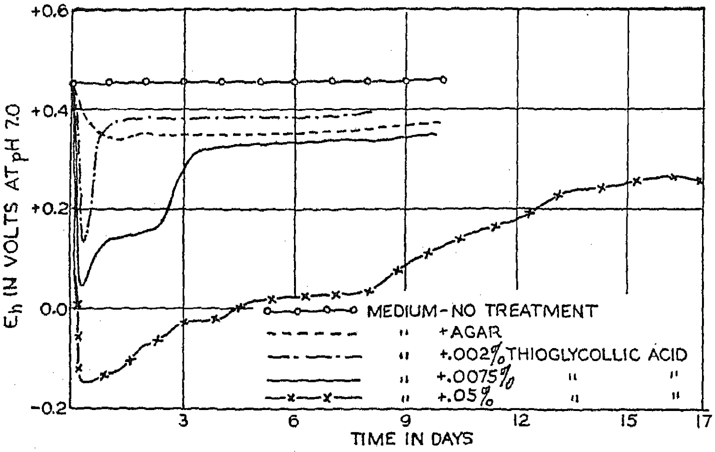

Fig. 3. Potential-time Curves of Sterile Mannitol Nitrate Medium with the Addition of Agar and Thioglycollic Acid

The effect of sterile powdered agar. Sterile powdered agar, when added to the mannitol-nitrate medium, gave marked stimulation to bacterial proliferation. The electrode potentials of this medium before and after the addition of 0.2 to 0.4 gram of the agar per 10 cc. quantities of the medium, indicate that this substance is not inert as an oxidation-reduction system. The presence of this amount of agar caused a fall of approximately 100 millivolts in the potential of the medium, a condition which prevailed for several days (fig. 3). The coiled electrodes were held at the surface of the mass of powdered agar in the bottom of the tube.

In the light of previous observations, the effects of the agar on bacterial growth in this purely synthetic medium would reasonably appear to be threefold: (*a*) the agar actually behaves as a mild reducing agent under these conditions; (*b*) certain impurities in the agar improve the nutritive balance of the medium, and (*c*) in a purely physical manner, due to its collodial nature, this material aids the organisms in making suitable oxidation-reduction adjustments themselves.

The last factor suggested is borne out in the experiments of Douglas, Fleming, and Colebrook (1917) in which it was found that porous substances such as cork, charcoal, sand, etc., when added to the medium greatly enhanced the growth of anaerobic bacilli.

Large versus small inocula. Unfavorable oxidation-reduction potentials seem to be less significant with large than with small inocula; and this is especially true when dealing with isolated individuals. The benefit derived from the associative action of microörganisms has been long recognized. Gillespie (1913), Webster (1925) Burnet (1925 and 1927), Valley and Rettger (1926), Quastel and Stephenson (1926), Dubos (1929) and others, working with a great number of different organisms, recognized that the organisms in some way assist each other in growth when in close proximity. To this beneficial association of microörganisms Churchman and Kahn (1921) applied the term "communal activity" and Robertson (1921), as a result of his studies on transplants of protozoa, introduced the term "allelocatalysis."

It is obvious that the bacteria themselves possess the faculty of making suitable adjustments of the potentials of the substratum within certain limits. It would naturally follow that the larger the inocula, the greater is this possibility. Under the conditions of the experiment referred to in table 1, the large inocula not only appear to be able to overcome conditions too oxidized but also conditions too reduced for optimum growth more effectively than do small inocula.

That large inocula can create more reduced conditions of their immediate environment by the respiratory activities of the protoplasm is not difficult to reconcile on a purely physiological basis. On the other hand, the benefit derived from this associative action under conditions too reduced for optimum growth is not so easily understood. The large inocula (10^{-1} and 10^{-2}) as shown in table 1 were able to withstand the low potentials produced by 0.05 per cent thioglycollic acid and proliferate, although growth was retarded for several days. Whether or not the large inocula were merely able to resist the reduced conditions and start growth after the potentials had risen to more favorable levels due to the auto-oxidation of the thio compound; or whether the bacteria themselves were able to exert some effect on a positive potential change is not definitely known.

The studies of Needham and Needham (1926) and Cohen, Chambers, and Reznikoff (1928) indicate that protoplasm is able to maintain a fairly constant potential within certain limits. More recently Plotz and Geloso (1930) have submitted some evidence to support the idea that bacterial cultures are able to exert an influence on a positive drift of potentials in substrata too reduced for optimum growth. It is well known that the cells of Nitrobacter are able to oxidize nitrite nitrogen as a source of energy and, at the same time, use the nitrite as a source of nitrogen in building protoplasm, a process which of necessity would involve a reduction of the nitrite before protein could be synthesized. This organism then must possess mechanisms for both oxidizing and reducing the nitrite compound.

Regardless of the theoretical explanations, it is seen experimentally that large inocula are able to withstand greater reduced conditions than small inocula. It would seem that the effect is in part due to the ability of the organisms to adjust the potentials in either direction, if not completely overtaxed by the adverse conditions of the system.

Limiting oxidation-reduction potentials. Recently several references have been made in the literature to the limiting oxidation-reduction potentials affecting bacterial growth. The studies of Fildes (1929), Knight and Fildes (1930) and Plotz and Geloso (1930) indicate that *Bacillus tetani* can initiate growth only within a definite potential range. Of course it is understood that these potential limits were operative for that substratum under investigation but not necessarily for all others, a point which should be properly emphasized.

A medium may be unfavorable for the best growth of the organisms for reasons other than an unfavorable oxidation-reduction potential. Under these conditions, it is reasonable to believe that slight deviations from the optimum oxidation-reduction range become of greater significance. This view is borne out in these studies by the data set forth in table 1.

The mannitol-nitrate medium, as such, did not support growth even when a 10^{-1} dilution of the organisms was used. Either of two changes in the medium made growth possible: (1) a change in the ion-balance by the addition of 0.015 per cent $CaCl_2$, or (*b*) the lowering of the potentials by the addition of thioglycollic acid. Either of these changes singly made growth possible whereas no growth occurred otherwise. On the other hand when the $CaCl_2$ was added and the system also reduced, an ideal situation seemed to be produced; good growth occurred throughout with high dilutions (10^{-7}) of the organisms. The limits of the potential range at which growth is possible with this organism seem to vary with the degree to which other factors are favorable to cell proliferation. A more suitable nutritive balance of the substratum appears to render it possible for the organisms to make wider adjustments of the potentials of the system.

Further, these studies indicate that lower potentials are required for rhizobia to initiate growth in the mannitol-nitrate medium than in the yeast-water mannitol medium. This is probably due to two factors: (*a*) the power of the nitrate medium to resist a change in potential when acted upon by the bacteria, and (*b*) the character of the nitrogen sources of the two media. In order to obtain results with the nitrate medium at all comparable with the yeast-water medium, the addition of 0.0075 per cent thioglycollic acid was necessary. This amount established a potential level in the nitrate medium well below that of any of the tubes of untreated yeast-water medium.

Greater reduction intensities seem to be required to reduce the nitrate preparatory to the synthesis of protoplasm than are required in the assimilation of the organic nitrogen of the yeast water. The transformation of NO_3 to NH_3 in the building of proteins would necessitate a reduction of the NO_3 by the organism.

THE OXIDATION-REDUCTION PROPERTIES OF CERTAIN MODIFICATIONS OF THE YEAST-WATER MANNITOL MEDIUM IN RELATION TO BACTERIAL GROWTH

The yeast-water mannitol medium, previously described, appears to be a very good medium for the growth of rhizobia. Growth is supported in high dilutions of the organisms and the total amount of growth is good. It is interesting to note the effects that certain modifications in the potentials of this medium have upon the ability of rhizobia to initiate growth as contrasted with the original untreated medium.

Effect of hydrogen peroxide alone. The hydrogen peroxide solution used contained 2.62 per cent actual H_2O_2. For the sake of convenience the various concentrations used were calculated as parts of this 2.62 per cent H_2O_2 solution per 1000 parts of medium. The hydrogen peroxide solution contained 0.20 grain of acetanilid per fluid ounce. This amount of acetanilid contained in the concentrations of hydrogen peroxide used was found to have a negligible effect upon bacterial growth.

A concentration of 1:3500 hydrogen peroxide solution was sufficient to inhibit the growth of a 10^{-1} dilution of rhizobia in the yeast-water mannitol medium. Further, it was found that the inhibitory effect produced by the peroxide in this medium was not dissipated rapidly under the conditions of these experiments.

Several sets of tubes of the yeast-water mannitol medium were prepared and divided into two series. A concentration of 1:3000 H_2O_2 was added to all the tubes of one series, and 1:3500 to the other at the beginning of the experiment. Subsequently, at intervals covering a period of eighteen days, one set of tubes of each series was inoculated with various dilutions of the organisms. Even eighteen days after the hydrogen peroxide had been added, these media failed to support growth of the organisms except in the case of the 10^{-1} dilution where the 1:3500 H_2O_2 had been added (table 2).

Electrode measurements on similar sterile systems indicate that a 1:3000 concentration of H_2O_2, when added to the yeast-water mannitol medium, produces a positive shift in the potentials of the system to a level of 490 to 500 millivolts positive to the normal hydrogen electrode. This potential level is well above that observed in any of the experiments with the sterile untreated yeast-water medium. Further, this potential level persisted over a period of time comparable to the duration of the growth experiments just referred to above (fig. 4).

Any attempt to explain the lasting effect of the hydrogen peroxide in this system, both on bacterial growth and on electrode potentials, becomes a matter of conjecture. Whether or not the peroxide persists as such or forms some more stable organic compound in the medium, is not known.

TABLE 2

The permanency of the effect of H_2O_2 *in yeast-water mannitol medium as related to the growth of Rh. trifolii 202*

TIME OF INOCULATION AFTER H_2O_2 WAS ADDED TO MEDIUM	CHECK—NO H_2O_2					CONCENTRATION OF H_2O_2 (2.62 PER CENT ACTUAL H_2O_2)									
						1:3,000 H_2O_2					1:3,500 H_2O_2				
	Dilutions of organisms														
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
12 hours later	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
24 hours later	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
3 days later	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
8 days later	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
12 days later	+	+	+	+	+	−	−	−	−	−	+	−	−	−	−
18 days later	+	+	+	+	+	−	−	−	−	−	+	−	−	−	−

These data remained unchanged after one month's incubation of the tubes.

Original inoculum was taken from a five-day fluid culture of the organism in the yeast-water mannitol medium.

Peroxide followed by thioglycollic acid. The following experiment was designed to determine whether or not the inhibitory effects of the concentrations of hydrogen peroxide, just referred to, were due to a poising of the medium at unfavorable potentials for cell multiplication, or to the killing of the organisms outright.

Several series of tubes of the yeast-extract mannitol medium were prepared and concentrations of hydrogen peroxide ranging from 1:3000 up to 1:4000 added to the tubes at the outset.

After a lapse of twelve hours all the series of tubes were seeded with various dilutions of the organisms. At subsequent intervals of twelve, twenty-four, forty-eight and eighty hours one set of tubes respectively of each series was reduced with 0.01 per cent thioglycollic acid (or 0.01 per cent cysteine). In some of the higher dilutions the organisms were killed, apparently by the hydrogen peroxide, during the course of the experiment. It was possible, however, to recover a number of the lower dilutions even after an exposure of eighty hours to these oxidized conditions (table 3).

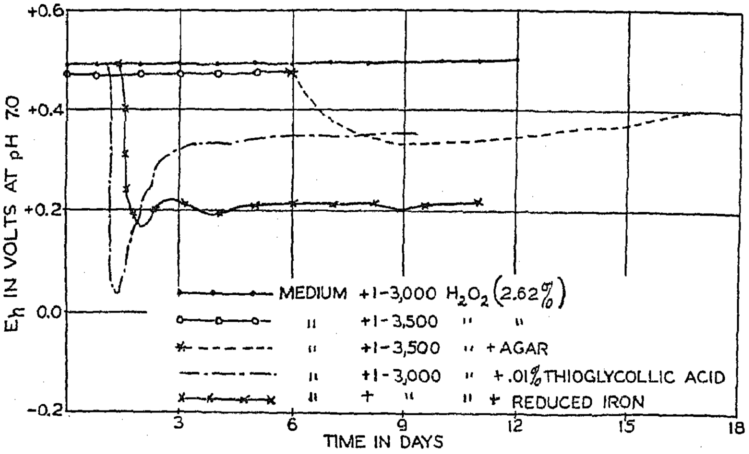

FIG. 4. POTENTIAL-TIME CURVES OF STERILE YEAST-WATER MANNITOL MEDIUM WITH THE ADDITION OF H_2O_2 AND SUBSEQUENT ADDITION OF THIOGLYCOLLIC ACID, REDUCED IRON, AND POWDERED AGAR

The potential-time changes produced by the introduction of the 0.01 per cent thioglycollic acid into the yeast-water mannitol medium containing the hydrogen peroxide were followed by electrode measurements under sterile conditions. Immediately after the addition of the reducing compound, a rather sudden low drop in the potential of the system was observed, followed by a rapid positive drift within thirty-six hours back to a region still some 150 to 175 millivolts negative, however, to that of original system. A slow positive change continued during the six days following (fig. 4).

It is evident in the light of these observations that the barely inhibitory concentrations of hydrogen peroxide exert their characteristic depressing effect on bacterial growth, in part at least, by poising the substratum at unfavorable potentials for cell multiplication.

Peroxide followed by other substances. In this experiment an endeavor was made to determine the effectiveness of certain other materials on the ability of the organisms to overcome unfavorable potentials induced by the addition of H_2O_2 to the yeast-water mannitol medium. Three concentrations of hydrogen peroxide were used: 1:3000, 1:3500, and 1:4000. The hydrogen peroxide was added to the tubes at the outset. Then, after twenty-four hours, the tubes were all seeded with the various dilutions of the organisms. After a lapse of another four hours, the materials such as sterile powdered agar, ground filter paper, fine sand, reduced iron and cysteine were added. Details of the set-up are given in table 4.

TABLE 3

The effect of H_2O_2 and the subsequent addition of thioglycollic acid to the yeast-water mannitol medium on the growth of Rh. trifolii 202

YEAST-WATER MANNITOL PLUS PARTS PER 1,000 OF H_2O_2	CHECK—NO THIOGLYCOLLIC ACID					0.0075 to 0.01 PER CENT THIOGLYCOLLIC ACID ADDED AFTER																			
						12 hours					24 hours					48 hours					80 hours				
	Dilutions of organisms																								
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
1:3,000 H_2O_2...	−	−	−	−	−	+	+	+	−	−	+	−	+	−	−	+	+	−	−	−	+	+	−	−	−
1:3,500 H_2O_2...	−	−	−	−	−	+	+	+	+	−	+	+	+	−	−	+	+	+	−	−	+	+	−	−	−
1:3,750 H_2O_2...	+	−	−	−	−	+	+	+	+	−	+	+	+	+	−	+	+	+	+	−	+	+	+	−	−
1:4,000 H_2O_2...	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−

The H_2O_2 solution (2.62 per cent actual H_2O_2), was added to the medium twelve hours before inoculation.

All tubes were inoculated at the same time and thioglycollic acid added at subsequent intervals as indicated.

Some advantages were noted under the conditions of the experiments resulting from the addition of such rather inert materials as sand and filter paper. The materials, probably by trapping small amounts of medium and preventing convection currents, render it possible for the organisms themselves to start growth within the interstices of these materials and spread to the surrounding medium.

The organisms seemed to derive greater benefit from the presence of the sterile powdered agar. This material not only affords greater physical aid to the bacteria in making their own adjustments due to its colloidal nature, but it also exerts a mild reducing effect upon the system itself under these conditions as shown by electrode measurements. Approximately 0.2 to 0.4 gram of the agar was introduced into 10 cc. of the medium (fig. 4).

TABLE 4

The effect of certain substances on the ability of the Rh. trifolii 202 to overcome unfavorable potentials induced by the addition of H_2O_2 to a yeast-water mannitol medium

YEAST-WATER MANNITOL MEDIUM + H_2O_2	PARTS OF H_2O_2 (2.62 PER CENT SOLUTION) PER 1,000 PARTS OF MEDIUM																	
	1:3,000 H_2O_2						1:3,500 H_2O_2						1:4,000 H_2O_2					
	Dilutions of organisms																	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Check	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
+ ground filter paper	+	−	−	−	−	−	+	+	−	−	−	−	+	+	+	−	−	−
+ fine sand	+	+	−	−	−	−	+	+	−	−	−	−	+	+	+	−	−	−
+ powdered agar	+	+	+	−	−	−	+	+	+	+	−	−	+	+	+	+	−	−
+ reduced iron	+	+	+	+	−	−	+	+	+	+	−	−	+	+	+	+	−	−
+ 0.01 per cent cysteine	+	+	+	+	−	−	+	+	+	+	−	−	+	+	+	+	+	−

The H_2O_2 was added to all the tubes; after twenty-four hours they were all inoculated; then after an elapse of some four hours, the various substances, powdered agar, etc., were introduced.

The addition of the cysteine and reduced iron to the yeast-extract medium containing the hydrogen peroxide produced somewhat similar results on bacterial growth. In each case some of the smaller inocula were unable to initiate growth probably due to injury incurred by exposure to the hydrogen peroxide before the addition of the reducing agents (table 4).

Approximately 1.5 to 2 grams of reduced iron were added per 10 cc. of medium in the experiment above. Under comparable conditions electrometric measurements showed that this amount of reduced iron was capable of producing a negative shift of some 300 millivolts in the potential of this medium containing 1:3500 H_2O_2 and was capable of maintaining this potential level over a period of several days. In making the potential readings, the electrodes were inserted in the clear medium just above the reduced iron after it had settled to the bottom of the tube (fig. 4).

Attempts to follow the potential-time changes produced by the 0.01 per cent cysteine under these conditions gave erratic results. The instability of this compound at pH 7.0 and the rapidity with which it is auto-oxidizable made attempts at comparable results in these aerobic media futile. Its reducing properties, however, were obvious in all cases. The thioglycollic acid appears to be much more stable. It is not so readily auto-oxidizable and does not require sterilization due to its sterile liquid state at the outset, being approximately a 13 N acid.

OXIDATION-REDUCTION POTENTIALS DEVELOPED BY CULTURES OF RHIZOBIA

The potential-time changes developed by cultures of rhizobia in the yeast-water mannitol medium were followed by electrode measurements in a manner previously outlined in this paper (fig. 1). The cultures were all incubated at 28°C. The experiments were initiated at pH 7.0 and hydrogen-ion determinations made again at the close of the experiment. These bacteria produce slight pH changes during their development in the yeast-water medium. The potential-time changes of the systems during the growth of these aerobic cultures are plotted against time in figure 5.

Rh. japonicum followed a potential-time course in a region very positive to that of the other three cultures of rhizobia. Certain discrepancies were noted between comparable electrodes in cultures of this organism. The two electrodes in the same culture were in perfect agreement at times, whereas, often on subsequent days, a discrepancy of some 30 to 40 millivolts between the two electrodes was observed. After growth had progressed for several days, one of the cultures was placed under a vaseline seal. The electrodes rapidly came into excellent agreement and remained so thereafter. The slow growth of these organisms and probably their uneven development accompanied by convection currents set up in certain regions of the tubes may have been responsible for the irregular behavior of the electrodes.

This positive zone of potential, characteristic of the *Rh. japonicum* culture, would appear to be due to the failure of the organisms to exhaust the dissolved oxygen of the system. At no time was pellicle formation evident. The oxygen up-take by the organisms is apparently slow. After the addition of the vaseline seal referred to above, a negative drift of potential obtained, below that of the aerobic culture, as the oxygen became exhausted by the organisms under the seal.

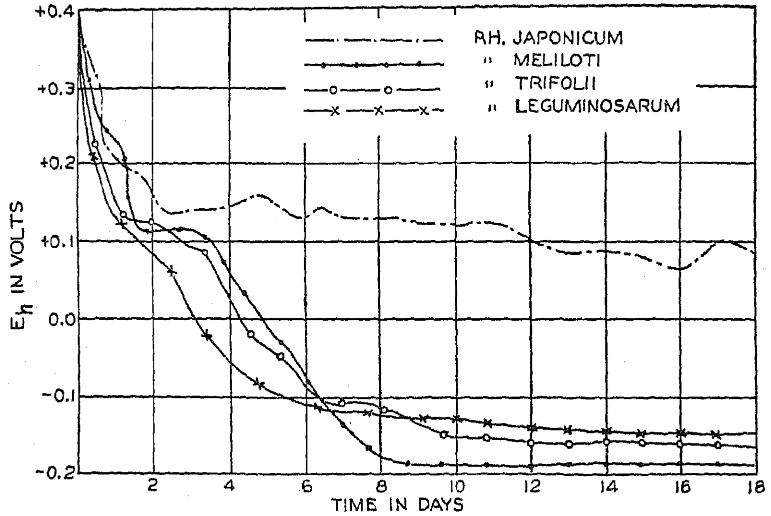

FIG. 5. POTENTIAL-TIME CURVES OF RHIZOBIA IN YEAST-WATER MANNITOL MEDIUM

Rh. trifolii, Rh. leguminosarum, and Rh. meliloti. These three aerobic cultures followed somewhat similar potential-time curves. The maximum low potentials attained, however, were all much lower than that of *Rh. japonicum.* During the first day a rapid negative potential drift occurred in all cases, followed by a slow continued drift over a period of several days (fig. 5).

It will be noted that *Rh. leguminosarum* and *Rh. trifolii* follow courses somewhat negative to *Rh. meliloti* during the first few days of growth; but *Rh. meliloti* attains the lowest final potential. The first two organisms appear to be more aerobic than *Rh. meliloti.* They grew throughout the medium at first but soon formed dense pellicles while the base of the tube almost completely cleared. *Rh. meliloti* grew diffused throughout the medium during the entire period forming a very thin pellicle only after nine days' incubation.

The greater demand for oxygen and early dense pellicle formation may account for the more negative potentials characteristic of *Rh. leguminosarum* and *Rh. trifolii* in the early growth stages. On the other hand, the magnitude of the growth throughout the medium in the case of *Rh. meliloti* during the entire growth phase may account for the final low potential attained in this case.

The growth of the organisms in the nitrate-mannitol never resulted in potentials as low as those attained in the yeast-water medium. The electrodes were also held much less constant in the nitrate medium. The differences obtained in the potentials of the two systems may be due to inherent differences in the two media themselves or they may be due to a less vigorous growth of the organisms in the nitrate medium.

Factors responsible for potential changes in bacterial cultures. The attempt to analyze the factors responsible for the negative drift of potentials characteristic of bacterial cultures is as old as the observation of the phenomenon itself. The fact that the reduction of dyes took place in the medium some distance out from the zone of bacterial growth suggested to Spina and to Rozsahegyi, as early as 1887 that diffused products of bacterial metabolism were probably active in the reduction processes. Sterilization, however, seemed to destroy the reducing properties of such systems. Cahen (1887) in the same year added another link to the chain of thought by suggesting that these diffusion products may be in the form of a gas and readily escape from the field of action, or may be readily oxidized upon being released from the bacterial cell. Hence, reduction of dyes would be evidenced only in the presence of living protoplasm, yet one of Spina's experiments showed very clearly that sterile nutrient gelatin itself was capable of reducing methylene blue.

More recently Barthel (1917) and Thornton and Hastings (1929) have emphasized the withdrawal of oxygen from the medium by the bacteria during the reduction of methylene blue in milk. The mere withdrawal of oxygen, however, will hardly account satisfactorily for all cases, especially where extremely low potentials approaching a hydrogen overvoltage are reached, far below the point where the last traces of oxygen have been exhausted.

Coulter (1928) and Coulter and Isaacs (1929) showed that cultures of *B. typhosus* developed potentials some 0.1 volt lower than were obtained in the sterile medium alone when deaerated with nitrogen. Plotz and Geloso (1930) contend that Coulter was unable to rid the medium of all the dissolved oxygen by deaeration with nitrogen, whereas the culture of *B. typhosus* carried the process to completion, thus accounting for the 0.1 volt more negative potential reached in the inoculated medium.

Plotz and Geloso (1930) devised a method for making electrode measurements in vessels hermetically sealed after deaeration of the system in order that the medium itself would dispense with the last traces of free oxygen. They conclude that bacterial growth has only a catalytic effect on the potential changes of the medium similar to that of platinum black in their experiments. The same low potentials were reached in all instances but more rapidly where bacteria were at work or when platinum black had been added to the medium. Working with seven different organisms they conclude further that the potential limits attained in bacterial cultures are not characteristic of the organisms developing therein, as all reach the same limit of reducing intensity. Hewitt (1930), on the other hand, showed that more highly reducing conditions were developed by *C. diphtheriae* than by cultures of haemolytic streptococci. Similar observations have been made with many other organisms.

It would appear that this situation is too complex to permit sweeping generalities or to warrant the assignment of the reducing properties observed in bacterial cultures to any isolated factor. To contend that all bacterial cultures develop the same low reducing potentials would naturally entail an analogous statement that all compounds resulting from bacterial metabolism possess similar electromotively active properties. It is difficult to reconcile on this basis the action of such metabolic products as hydrogen sulfide and peroxide, the one a reducing compound and the other an oxidizing compound.

In the course of these studies on rhizobia certain data were obtained which, it is believed, lend some information concerning the factors pertinent to the reducing properties of growing cultures. After a growth period of three days, one of two comparable cultures of *Rh. meliloti* was placed under a vaseline seal. For a few days after the addition of the seal, this system fell in potential somewhat more rapidly than the aerobic culture. As growth progressed, however, the potential fall in the aerobic tube surpassed that of the anaerobic tube and continued more negative (some 120 millivolts) until the close of the experiment (fourteen days). If the change in potential were to be attributed wholly to the exhaustion of oxygen from the system, then the presence of the seal, it would appear, should have facilitated the development and maintenance of a more negative potential.

Data were obtained also which indicate that a bacteriological system is in a more reduced state subsequent to bacterial growth and sterilization than that of the original medium. Fresh tubes of medium were prepared and initial potential readings made. The tubes were then heavily inoculated with *Rh. trifolii*, *Rh. meliloti*, and *Rh. leguminosarum*. After eight days' incubation the cultures were autoclaved at 15 pounds for twenty-five minutes, rapidly cooled to 28°C. and clean electrodes again inserted. The sterilized cultures on an average tested some 100 to 150 millivolts more negative than the original sterile medium. The autoclaved culture underwent gradually a positive potential drift of approximately 20 millivolts daily for the first four days following sterilization, indicating the presence of some auto-oxidizable substance that had not been destroyed by sterilization.

The controversies arising from the various attempts to explain the reducing properties of bacterial cultures may be reasonably compromised by remembering that bacterial cultures are specific entities. Even though many factors may be involved in the reducing properties of bacterial cultures as a group, the importance of any one factor or of a combination of factors for a given system must depend upon the specific character of the system in question.

THE BEHAVIOR OF BACTERIA TOWARD ATMOSPHERIC OXYGEN AS RELATED TO OXIDATION-REDUCTION POTENTIALS

Allyn and Baldwin (1930) reported that rhizobia in shake-agar cultures develop distinct growth zones at various levels in the tubes depending upon the oxidation-reduction character of the medium. Similar growth levels may be observed with rhizobia in liquid media, if care be taken not to disturb the cultures during growth. It was noted that, as the liquid media were made more reducing, the first signs of growth appeared correspondingly nearer the surface of the medium. The converse was true when the potentials were made more oxidizing.

In the experiments with the yeast-water mannitol medium to which hydrogen peroxide had been added in amounts just short of the inhibition of a 10^{-1} dilution of rhizobia, growth appeared first at the very bottom of the tube and diffused up as reducing conditions were set up by the bacteria. Ultimately, as the potentials of the system reached a low level due to bacterial growth, pellicle formation occurred and the bottom of the tube began to clear. The system, as shown by electrode measurements, passed through a potential range from $E_h = +0.490$ volt at the outset to $E_h = +0.050$ volt, at which time pellicle formation was evident with *Rh. trifolii*.

In the case of the mannitol-nitrate liquid medium, previously referred to, as the tubes of medium were reduced progressively with increasing amounts of thioglycollic acid, the first signs of growth appeared correspondingly nearer the surface of the medium. When 0.002 per cent of the reducing compound was added, growth appeared first at the bottom of the tube with a 10^{-1} dilution of *Rh. trifolii*. With the addition of 0.005 per cent thioglycollic acid, growth first appeared about the middle of the tube; with 0.0075 per cent, about 1 cm. below the surface, and with 0.01 per cent, very close to the surface of the medium.

The smaller inocula initiated growth at zones somewhat lower than those just described for the 10^{-1} dilution under similar conditions. This difference is probably due to the lesser reducing properties of the smaller inocula themselves and the rise in the potential of the substratum by the auto-oxidation of the thio-compound during the greater lapse of time necessary for the smaller inocula to produce visible signs of growth.

In the case of rhizobia in the yeast-water medium, signs of surface growth could be predicted with surprising accuracy by means of the potentiometer. Signs of pellicle formation appeared with *Rh. leguminosarum* in undisturbed cultures at $E_h = +0.110$ to +0.090 volt; with *Rh. trifolii* at +0.060 to +0.040 volt; and with *Rh. meliloti* at +0.010 to −0.010 volt.

Rh. japonicum never formed pellicles, and as indicated in figure 5 this culture followed a potential-time course in a region very positive to the other three organisms. The oxygen up-take of *Rh. japonicum* is evidently not large. It appears evident that the phenomenon of pellicle formation with rhizobia is an expression of an oxygen relationship, or of the degree to which the organisms are aerobic in character.

SUMMARY

A vacuum-tube potentiometer circuit is described for avoiding polarization in making electrometric measurements of oxidation-reduction potentials of biological systems.

The studies indicate that the oxidation-reduction potentials of the medium exert a decisive influence on the ability of certain aerobic bacteria to initiate growth.

Two basic media were studied: a yeast-water mannitol and a mannitol-nitrate medium. The first medium was found to be the more reducing in nature and to support growth with high dilutions of the organisms. The mannitol-nitrate medium permitted growth in similar dilutions only after the potential was properly reduced. It was possible to poise the yeast-water mannitol medium with H_2O_2 at potentials sufficiently high to inhibit growth completely, and yet recover the organisms after eighty hours' exposure to these conditions, by reducing the potentials of the systems.

Commercial agar was found not to be an electromotively inert substance. The agar not only appears to afford physical advantages to the organisms in initiating growth due to its colloidal nature, but also to act as a mild reducing agent under the conditions of these experiments.

Large inocula possess greater ability to overcome unfavorable oxidation-reduction conditions than small inocula. This seems to hold true in media either too oxidized or too reduced for optimum growth.

The oxidation-reduction potentials which limit bacterial growth seem to be somewhat dependent on the other factors characteristic of the medium. A more suitable ion-balance or a more available nitrogen source, for example, appears to facilitate wider adjustments of unfavorable potentials by the bacteria themselves.

Potential-time courses developed by rhizobia have been followed by electrode measurements. The factors responsible for the negative drift of potentials in the bacterial cultures are discussed.

The behavior of the rhizobia toward atmospheric oxygen is determined largely by the oxidation-reduction character of the medium, as indicated by the growth levels established by the organisms in media possessing different oxidation-reduction properties.

Pellicle formation in liquid media seems to be definitely correlated with the oxidation-reduction potentials of the system in the case of rhizobia. The bacteria collect at the surface in a pellicle as the potential of the medium falls, due to their metabolic activities.

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THE ANOMALOUS BEHAVIOR OF THE TUBERCLE BACILLUS AS A GRAM-POSITIVE ORGANISM WITH HIGH RESISTANCE TO GENTIAN VIOLET

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In a review of a monograph by the present authors (Stearn and Stearn (1928)) Wilson (1929) included the statement that, on the basis of the thesis put forward by them that the bacterial cell behaves as an equilibrated system of two classes of ampholytes the one more acidic in nature than the other, "no explanation is afforded for the anomaly that the tubercle bacillus, though Gram-positive, has yet a high resistance to the bacteriostatic effect of gentian violet." In the present paper data are presented from which the explanation of this anomaly, which is apparent rather than real, may be adduced.

Not only the tubercle bacillus, but also other acid-fast organisms, present apparent anomalies in behavior, in that though they are Gram-positive, their resistance to gentian violet is of a higher order than is usual with other members of the Gram-positive class of organisms and reaches that of many of the Gram-negative strains. A closer study of this anomaly offers an excellent opportunity for testing any theory which attempts to explain the Gram reaction on the basis of a general mechanism which should correlate staining behavior with physiological behavior toward dyes.

Experimenters have noted considerable divergencies among strains of tubercle bacilli in their staining properties and their dye sensitiveness. Churchman (1912) found, by inoculation on divided plates, that, of four strains all of which were Gram-positive, two strains were inhibited by 1:100,000 gentian violet, one strain was not, while the results with the fourth were not definite. Suyenaga (1920) found, in general, that the less acid-fast organisms were more susceptible to the action of dyes. He noted, for example, that on glycerol agar 1:1,000 gentian violet caused no inhibition of growth in the case of a saprophytic non-virulent strain of human tubercle bacillus, and that strains of avian and frog tubercle bacilli were completely inhibited by 1:100,000 gentian violet, while *Bacillus leprae* was completely inhibited by a dye concentration of 1:10,000.

Petroff and Steeken (1930) used a glycerol-egg medium containing 1:30,000 gentian violet for dissociating the tubercle bacillus.

The present authors found strain 115 (American Type Culture Collection) to grow at gentian violet concentrations ranging from 1:40,000 to 1:200,000 on glycerol agar after three weeks' incubation. Whitman's strain (1926), which he obtained by culturing a Koch-Novy strain on bone marrow agar, grew well in concentrations of 1:10,000. This, a defatted strain, grows readily on nutrient agar and from such agar slants is less Gram-positive than either *Bacillus subtilis* or *Staphylococcus aureus*. If decolorized with acetone the rods are either Gram-negative or contain minute Gram-positive granules. While it is still acid-fast if the carbol fuchsin is gently heated for five minutes, this strain is non-acid-fast if the stain be applied cold for fifteen to twenty minutes.

While some of these divergencies may be due to difficulty in measuring the inoculum and in preventing the transfer of clumps, this cannot be the entire explanation; and variability both in staining and resistance which, while found to some extent in many if not all organisms, is so striking in the tubercle bacillus, as to indicate factors at work here which are either absent in many bacterial species or else much less effective. The present study indicates two things. In the first place the mechanism of dye resistance may not be the same as in many bacteria, and in the second place the Gram character is probably associated very closely with the acid-fastness.

I. DESTRUCTION OF GENTIAN VIOLET BY BACILLUS TUBERCULOSIS

All of the strains of this organism we have studied have, in a very marked form, the ability to decolorize gentian violet if the dye concentration will permit growth at all. Often, in dye concentrations from 1:10,000 to 1:200,000 the degree of decolorization is closely related to rapidity of growth. In cases of the more slowly growing strains, at dye concentrations of 1:100,000 to 1:200,000 the decolorization can be noted from the very beginning of an appearance of growth. This decolorization is not merely a removal of dye from the medium by adsorption onto the bacterial mass. The waxy pellicle, even in 1:10,000 gentian-violet glycerol broth or agar does not become stained or darkened in the least, and all evidence that purple dye has ever been present vanishes. This behavior is in direct contradistinction to that of the colon-aerogenes group which, during growth, may, in alkaline media, remove a portion of the dye from the medium, the bacteria themselves becoming intensely stained (Stearn and Stearn (1923)). The tubercle bacilli, taken from these decolorized media, show no staining when examined microscopically.

This striking action on the dye does not result in any great alteration in the bacteria, as both the pellicle and the small sediment in the tubes in which the dye has been decolorized showed, when examined microscopically, Gram-positive, acid-fast tubercle bacilli.

The fundamental nature of the change wrought on the dye was shown by the following experiments. A strain of *Bacillus subtilis* inhibited by 1:4,000,000 gentian violet at pH values down to 6 was inoculated into tubes containing clear sterilized gentian-violet broth which had previously been decolorized by the tubercle bacillus, and which had been diluted to desired values of decolorized dye concentration with sterile nutrient broth. The final diluted broth would have given gentian-violet concentrations of 1:500,000; 1:1,000,000 and 1:2,000,000. In all cases there was luxuriant growth in twenty-four hours. There was considerable growth in twenty-four hours even in tubes adjusted to concentrations of decolorized dye as high as 1:40,000 to

1:80,000. In all cases stained smears revealed long chains of typical Gram-positive rods.

Here, then, we see a semi-specific mechanism for resistance to gentian violet. It is more than probable that the protecting waxy coat furnishes an auxillary protection against the dye. This view is strengthened by the fact that, while many strains of tubercle bacilli have been found to grow in gentian violet 1:1,000 (Suyenaga (1920)) to 1:10,000 (see above), our strain of *M. smegmatis* was inhibited by 1:100,000 gentian violet. Churchman also reports that growth of this organism is "delayed" at 1:100,000 of this dye. These results are of interest as the organism is, though Gram-positive, less acid-fast than a virulent culture of the tubercle bacillus.

II. DEPENDENCE OF GRAM CHARACTER ON THE ACID-FAST CONDITION

When an ordinary strain of acid-fast tubercle bacillus is stained by the Gram technic it retains the primary stain. There is a good deal of question, however, whether removal of the material which causes this acid-fastness will not also yield a product which is no longer Gram-positive. Aronson (1910) found this to be the case. He was able, by treatment with trichlorethylene, to obtain smears of tubercle bacilli which were not only non-acid-fast but were also Gram-negative, not even showing Much granules. This behavior he showed clearly was *not* general for Gram-positive organisms, since both staphylococci and streptococci, treated in a similar manner, remained Gram-positive.

Whitman (1926) claims that his strain of "defatted" tubercle bacilli has, to a large extent, lost its Gram-positiveness with its loss of acid-fastness. Our experience with this strain grown on nutrient agar was, as stated above, that, compared with *B. subtilis* or *S. aureus*, it is only extremely weakly positive if not negative, since the use of acetone as a decolorizer gives negative smears. When grown on glycerol media both the strong acid-fastness and the strong Gram-positiveness of the ordinary tubercle bacillus strains are restored. That the glycerophilism of the group of acid-fast bacilli is related to wax production has been shown by

Long and Campbell (1922); while Frouin (1921) has found that the lipin content of the tubercle bacillus is greatly reduced if, in place of glycerol, sugars are used in the media.

Table 1 indicates the difference in Gram behavior between the Whitman strain and an ordinary acid-fast strain. Slides were prepared with smears of both these strains as well as with one of the Gram-negative *B. coli* and one of the Gram-positive *B. subtilis* for reference. Column 1 in the table describes the treatment to which the slide was subjected, while the other columns give the results of subsequent application of the Gram technic.

This table brings out the fact that the defatted strain differs decidedly in its Gram behavior from the ordinary strain. Acidic substances, which tend to make *B. subtilis* appear negative, have the same effect to even a slightly greater degree on the Whitman defatted strain, whereas the ordinary acid-fast strain remains unaffected by such treatment.

TABLE 1

TREATMENT OF SLIDE	SUBSEQUENT GRAM CHARACTER			
	B. coli	*B. subtilis*	*B. tuberculosis*	
			Whitman strain	Ordinary strain
Control	−	+	+	+
20 cc. 1 per cent HCl in ether-alcohol poured over slide	−	−	−	+
Slide soaked one minute in 1 per cent HCl in ether-alcohol	−	−	−	+
Slide soaked five minutes in acetic anhydride	−	±	−	+

SUMMARY

In connection with the apparent anomaly of the tubercle bacillus being Gram-positive and yet highly gentian-violet resistant, the following facts have been shown:

1. Besides the probable protecting action against gentian violet of the waxy coat of ordinary tubercle bacilli, this organism seems to have a decomposing action on the dye, changing it to

a form which is found to be non-inhibitive to *B. subtilis* even at high concentrations.

2. The Gram behavior of the defatted organism is entirely different from that of the ordinary acid-fast organism.

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THE DECOMPOSITION OF PROTEINS BY MICROÖRGANISMS WITH PARTICULAR REFERENCE TO PURIFIED VEGETABLE PROTEINS[1]

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Investigations concerned with the decomposition of proteins by microörganisms have practically all been limited to the proteins of animal origin, such as milk proteins and proteins of the blood, muscle, eggs, etc. However, as regards decomposition of proteins in soil and in compost, plant proteins are more important, since considerably larger quantities of these find their way into the soil. It is, therefore, essential to determine to what extent plant proteins, as compared with animal proteins, are decomposed by the microörganisms, and whether the course of their change and the products formed are the same or are essentially different.

The literature is full of references to the fact that in the case of certain plant residues, such as leaves and needles of trees, straw, etc., very little nitrogen is liberated as ammonia in the course of decomposition, especially during its early stages. This may either be due to the resistance of the specific proteins to decomposition, or to the formation of complex combinations between the proteins and other plant or microbial constituents which render them more resistant to decomposition, or to the influence of the carbohydrates present in the same material,—the carbohydrates being able to reduce the liberation of the nitrogen in a mineralized form by suppressing the rapid decomposition of the protein or by favoring such extensive synthesis of microbial cell substance as to reduce the liberation of ammonia.

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This study was undertaken with the purpose of throwing light upon the first problem, namely, the comparative rapidity of decomposition of purified plant and animal proteins by microörganisms, primarily by forms isolated from the soil. Several proteins were used for this purpose. The purified plant proteins were kindly placed at the disposal of the authors by the late Dr. T. B. Osborne. The casein was purified *nach Hammarsten*. The fibrin, gelatin and albumin were commercial preparations. Certain amino-acids (glycocoll, alanine, asparagine) were also used in this study, for comparative purposes.

METHODS

The medium generally employed in these studies contained the following mineral constituents:

	Grams
K_2HPO_4	1.0 or 3.0
$MgSO_4 \cdot 7H_2O$	0 or 0.5
NaCl	0.1 or 0.5
$FeSO_4$	0 or 0.01
Distilled water	1,000

The proteins were used as the sole sources of energy and nitrogen in most cases. In a few instances glucose was used as an accessory source of energy. The liquid medium was distributed in 100 cc. amounts in 250 cc. Erlenmeyer flasks. In the presence of magnesium sulfate the large amounts of ammonia which were produced by the microörganisms led to the formation of crystals of magnesium-ammonium phosphate which were relatively insoluble in the medium and consequently interfered with the complete recovery of the ammonia in the analyses. In most cases the magnesium salt was left out of the medium and no influence on growth was observed as a result.

The proteins were added to the media in amounts of either 0.5 or 1.0 per cent. Where fibrin, albumin, zein, gliadin or edestin were used, the desired amounts of the air-dried proteins were weighed out and added to the flasks of liquid medium. The casein was brought into solution before addition to the medium, by dissolving each gram in 8 cc. of 0.1 N NaOH. The gelatin was also dissolved in the liquid medium before being added to the flasks. The reaction of the media was adjusted to neutrality. Incubation of cultures was carried out at 27 to 28°C.

The analyses of the cultures were carried out as follows: The cultures were filtered through weighed filter papers and the residual protein and the cellular material on the paper were washed with distilled water. The filtrate and washings were added together and made up to a definite volume. The residual protein and cellular material was dried at 75 to 85°C. to constant weight, and total nitrogen determined by a modified Kjeldahl method. The filtrate was analyzed for total nitrogen, amino nitrogen and ammonia nitrogen. The ammonia nitrogen was determined by a modified Folin aeration method. To 25 cc. of the filtrate was added 5 cc. of 40 per cent Na_2CO_3 solution, 5 cc. of 20 per cent NaCl solution and a small amount of crude oil to prevent foaming. The tube containing the liquid was placed in a water bath at 65°C. and attached to an aeration system and the volatilized ammonia was trapped in standard 0.05 N H_2SO_4. Aeration was continued for four to six hours. After the ammonia had been removed, the liquid was neutralized with acetic acid, made up to volume, and the amino nitrogen determined by the Van Slyke micro method. The hydrogen-ion concentration of the culture was determined by the colorimetric method. Casein nitrogen was determined on portions of the filtrate by the method of Sherman and Neun (1916). The solution was poured into 25 cc. of 20 per cent potassium sulfate solution and then 5 cc. of 0.2 N HCl was added. After standing for one hour the precipitated casein was separated from the liquid by filtration and the nitrogen content was determined. Glucose was determined by the Bertrand method.

EXPERIMENTAL

Utilization of glycocoll and casein by microörganisms. It has been shown by Waksman and Lomanitz (1925), and by others previously, that the ability to attack both amino acids and proteins may not be common to one bacterium. An organism like *Pseudomonas fluorescens* may decompose amino acids actively but does not attack casein to any extent. On the other hand, a bacterium like *Bacillus cereus* may make little or no growth on amino acids but develops well on casein. The following experiment adds to this evidence.

TABLE 1

Growth of soil organisms on casein and glycocoll media

SOURCE OF ORGANISM	ORGANISM	GROWTH ON GLYCOCOLL	GROWTH ON CASEIN
Casein agar	No. 1	−	+
	No. 2 (*B. mycoides*)	−	+
	No. 4 (*Ps. caudatus*)*	−	+
	No. 5 (*B. subtilis*)	−	+
	No. 6	+	+
	No. 7 (*Sarcina lutea*)	−	+
	No. 8	−	+
	No. 9 (*Ps. fluorescens*)	−	−
Glycocoll agar	No. 10	+	+
	No. 11	+	+
	No. 12	+	+
	No. 13	+	±
	No. 14	−	−
	No. 15	−	+
Stock cultures	*Cellulomonas fimi*	−	−
	B. stutzeri	−	−
	Sarcina lutea	−	+
	B. subtilis	−	+
	Ps. fluorescens	+	Slight
	Act. bobili	−	+
	Act. viridochromogenus	+	+

* The organism referred to as No. 4 was an active proteolytic non-spore forming bacterium, producing abundant orange yellow mucoid growth on nutrient agar and undoubtedly the same as the bacterium referred to by Conn (1919) as an orange gelatin-liquefying bacterium and named *Pseudomonas caudatus*.

Two solid media were prepared, one containing glycocoll as the source of carbon and nitrogen, the other containing casein. Dilutions of soil were plated out using these solid media and some of the bacteria which developed were isolated from colonies and transferred for the following studies. These bacteria were inoculated upon two solution media to determine their ability to attack casein and glycocoll respectively (table 1).

The results show that those organisms which were isolated from glycocoll plates practically all grew on glycocoll in the liquid medium and also grew on casein. However, those which were isolated on casein agar grew well on the casein solution but only one of the nine cultures developed on glycocoll. Among the few laboratory cultures which were also tested on both media, casein was more commonly available than glycocoll. Many fungi tested grew very well in the casein medium. They were not tested on glycocoll, however.

It is logical to expect that the ability to attack glycocoll and use it as the sole source of energy, as well as of nitrogen and carbon, would be a property related to fewer organisms than the ability to attack casein which, by its complexity, furnishes a greater variety of molecular groups which organisms could utilize. The fact that organisms can live and develop with glycocoll as their exclusive source of energy, carbon and nitrogen indicates an ability on the part of the organism to synthesize a great variety of organic compounds in order to construct the numerous constituents of the cell. Whether the microörganisms are capable of utilizing some of the amino acids for the building of their protoplasm or whether the protein molecule must be at first broken down completely to ammonia before the microörganism synthesizes its own proteins has received much consideration in the past (Abderhalden and Rona (1910); Czapek (1901, 1902a, 1902b); Waksman (1917)).

The fact that many organisms will grow on casein and not on glycocoll is no proof that these organisms would not develop on other amino acids. It is quite reasonable to suppose that some of the other more complex amino acids would be attacked more readily.

Products resulting from the decomposition of casein. Some of the organisms which grew well in the casein medium were grown in casein solution in order to obtain information concerning the products of decomposition. The results presented in table 2 show that the protein was quite completely decomposed in all cases but one, namely, in the presence of *Actinomyces bobili*, where only one-third of the casein had disappeared. In practically all cases much more nitrogen was recovered as ammonia than in the amino form; however, varying amounts of amino nitrogen were found in all of the cultures. The total nitrogen recovered in the inoculated media was in most cases considerably less than that present in the control. In view of the marked increases in alkalinity, it seemed likely that considerable losses of ammonia might have occurred. Similar evidence is gathered from the data in figure 1, where the results of another experiment are reported. The total nitrogen of the cultures decreased during the advanced periods of growth of the organisms and the ammonia decreased at the late periods when it would be expected to increase. In all cases of suspected loss of ammonia, the reaction was distinctly alkaline.

TABLE 2

Formation of amino and ammoniacal nitrogen from casein by soil organisms

Milligrams of nitrogen in culture

ORGANISM*	pH	NH_3-N	NH_2-N	N IN GROWTH	CASEIN N	NITROGEN ACCOUNTED FOR	TOTAL N IN CULTURE
Control	7.4				60.8	60.8	66.9
No. 1	8.6	22.4	9.5	7.2	9.3	48.4	52.4
No. 2 (*B. mycoides*)	8.6	21.9	6.0	5.0	5.3	38.2	52.9
No. 4 (*Ps. caudatus*)	8.6	21.9	1.3	4.6	12.7	40.5	45.9
No. 5 (*B. subtilis*)	8.6	19.1	3.1	4.2	4.4	30.8	49.6
No. 6	8.8	16.0	11.0	8.2	11.5	46.7	54.8
Sarcina lutea	8.6	9.5	13.6	3.1	5.5	31.7	66.4
B. subtilis	9.0	11.8	2.8	6.1	7.2	27.9	48.8
Act. bobili	8.2	7.3	2.2	2.9	45.9	58.3	66.7
Act. viridochromogenus	8.8	14.9	4.2	7.4	17.0	43.5	52.8
Act. violaceus-ruber	9.2	18.8	4.2	5.5	5.7	34.2	40.5

* Cultures fifty-three days old at time of analysis.

Certain interesting relationships also appear from the determinations of the amino nitrogen in the cultures. In most cases this nitrogen forms only a small portion of the amount of nitrogen actually recovered as ammonia. Exceptions occur in the culture of *Sarcina lutea* and in the early stages of growth of most of the other cultures where the amounts of amino nitrogen even exceeded at times the amounts of ammonia nitrogen. Those results seem to confirm certain of the observations of Waksman and Lomanitz (1925) which indicated that in the early stages of protein decomposition, some microörganisms tend to accumulate considerable amounts of amino acids. During more prolonged incubation, the compounds are further attacked and the nitrogen appears as ammonia. The results leading to high amino nitrogen with *Sarcina lutea* at advanced stages of decomposition can be explained by the slow attack on some of the amino groups by the organism. The largest quantity of amino nitrogen found was

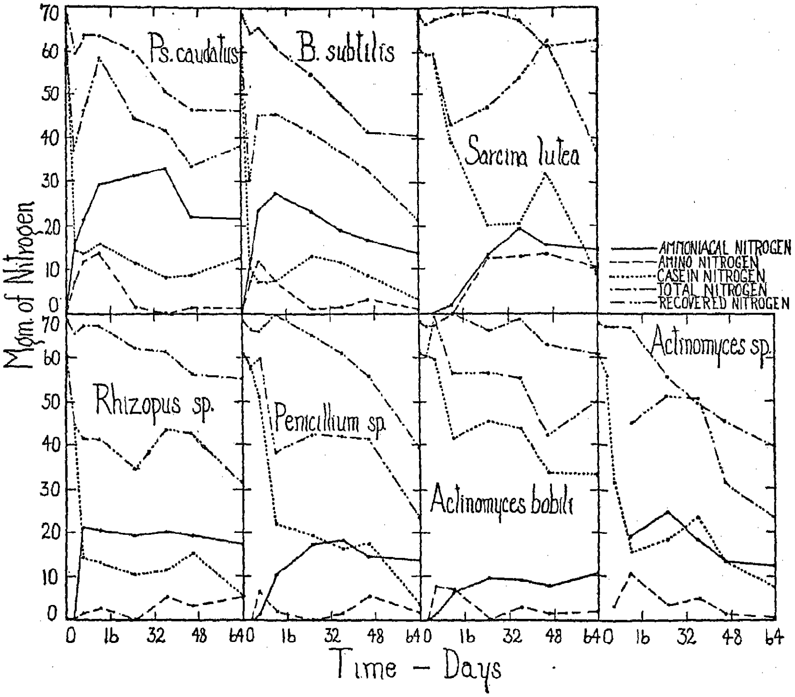

FIG. 1. PRODUCTS OF DECOMPOSITION OF CASEIN BY MICROÖRGANISMS
Recovered nitrogen is the sum of the ammoniacal nitrogen, amino nitrogen, casein nitrogen and nitrogen in microbial growth.

20.1 per cent of the total original nitrogen; the largest amount of ammonia nitrogen recovered was 48.5 per cent of the total original nitrogen. If allowance was made for the amounts of nitrogen probably lost as ammonia by volatilization, the actual production of ammonia would be much greater (in some cases more than 75 per cent of the nitrogen initially present).

The results in figure 1 also point to the formation in certain cases of considerable quantities of various forms of nitrogen other than ammonia or α amino nitrogen, since the nitrogen recovered as ammonia, amino nitrogen, casein and in growth of the organisms in the cultures does not account for nearly all of the nitrogen which was present in the solution. Particularly striking in this respect are the cultures of the fungi.

The curves for recovered nitrogen show a pronounced drop in the early stages of decomposition and later more closely approach the curves of total nitrogen. This suggests that, in the early stages of decomposition of the casein, considerable amounts of substances were produced which were intermediate between the casein and amino acids; some of these compounds were further decomposed during more extended incubation resulting in more complete recovery of the nitrogen in the various fractions which were determined.

In most cases the casein was practically all decomposed during the first few days; the continuous formation of ammonia must have been at the expense of the amino nitrogen or other forms of nitrogen which were not determined.

In order to establish to what extent the ammonia becomes volatilized from the cultures, a series of flasks containing casein medium and inoculated with pure cultures of various organisms, was attached to an aeration system in the incubator so that a stream of air, free from ammonia, passed above the liquid in the flasks and then through standard 0.1 N H_2SO_4 solution. The amounts of ammonia absorbed by this acid were then determined. The results presented in table 3 show that in a period of forty-five days one-sixth to one-third as much of the ammonia was recovered in a volatilized form as was found in the culture itself. When the amounts of volatilized ammonia are added to the total nitrogen in the cultures, it is found that almost as much nitrogen was recovered from the inoculated media as from the control. A small amount of nitrogen was still unaccounted for; it represented at most 6.1 per cent of the nitrogen in the control.

In a series of other experiments on the decomposition of casein, both alone and in the presence of various amino acids, it was found that appreciable quantities of ammonia are volatilized, increasing with age of culture and alkalinity of medium (fig. 2). This may amount to as much as one-third of the total ammonia formed by the organism or over two-thirds as much nitrogen as that recovered as ammonia in the culture. When this is taken into consideration, nearly all of the nitrogen in the culture can be accounted for. The losses of nitrogen not accounted for may be due to volatilization of nitrogenous compounds other than ammonia; formation, by cultures, of resistant nitrogenous compounds which were not detected in the Kjeldahl determination; volatilization of gaseous nitrogen. Although the weight of experimental evidence tends to discredit the possibility of loss of gaseous nitrogen in the absence of nitrate or nitrite, the possibility still exists that some nitrogen may thus escape from media containing an abundance of various organic nitrogenous compounds. These results may enable one to state that most of the nitrogen

TABLE 3

Volatilization of ammonia from cultures of microörganisms in casein media

Incubation, forty-five days

CULTURE	pH	TOTAL N IN CULTURE	NH_3-N		TOTAL N IN CULTURE, PLUS VOLATILIZED N
			In culture	Volatilized	
		mgm.	*mgm.*	*mgm.*	*mgm.*
Control	7.2	60.1			60.1
Ps. caudatus	8.7	51.9	28.0	4.6	56.5
B. subtilis	8.6	52.1	19.6	6.9	59.0
Rhizopus sp	8.7	53.3	19.8	3.6	56.9
Act. violaceus-ruber	8.8	49.5	24.9	6.9	56.4

lost from the cultures was volatilized as ammonia, due to the increase in alkalinity of the medium.

Decomposition of animal and plant proteins. A comparative study has been made of the decomposition of animal and plant proteins by pure cultures of microörganisms. For this purpose, definite amounts of protein (500 mgm.) were placed in 250 cc. Erlenmeyer flasks containing 100 cc. of medium with the necessary minerals. The cultures were sterilized, inoculated and incubated for varying periods of time (table 4).

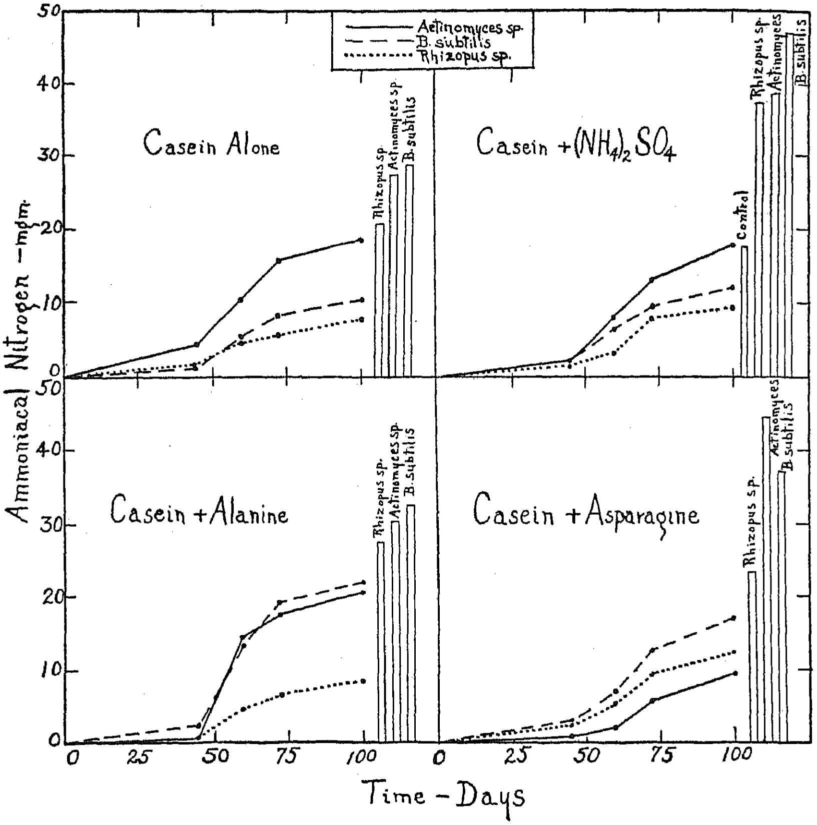

FIG. 2. VOLATILIZATION OF AMMONIA BY MICROÖRGANISMS IN CASEIN MEDIA

Lines indicate amounts of ammoniacal nitrogen volatilized; columns indicate amounts of ammoniacal nitrogen in the culture media after incubation for one-hundred days.

TABLE 4

Comparative decomposibility of plant and animal proteins by soil organisms

PROTEIN	ORGANISM	AGE OF CULTURE	pH	TOTAL N IN SOLUTION	N RECOVERED AS PROTEIN	NH_3-N		N IN MICROBIAL GROWTH AND IN THE INSOLUBLE PART OF THE PROTEIN
						Found	Corrected	
		days		*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
Casein......	Control		7.5	60.1	45.6			0.8
	Ps. caudatus	9	8.2	57.7	10.8	21.5	22.5	2.1
	Ps. caudatus	39	8.4	52.9	6.9	37.8	44.9	0.9
	B. subtilis	9	8.2	63.3	13.3	15.8	15.8	1.3
	B. subtilis	39	8.8	49.3	4.0	23.6	32.6	2.6
	Act. violaceus-ruber	9	8.2	58.8	16.4	14.6	14.6	1.6
	Act. violaceus-ruber	39	9.2	46.9	5.3	22.0	33.2	2.8
	Rhizopus sp.	9	7.8	58.6	17.4	16.2	16.2	2.4
	Rhizopus sp.	39	9.0	52.5	5.3	18.8	24.0	3.1
Gelatin.....	Control		7.2	96.8				0.8
	Ps. caudatus	9	8.4	89.2		21.0	27.9	1.4
	Ps. caudatus	21	8.8	71.1		25.1	42.3	9.2
	B. subtilis	9	8.8	87.2		41.5	47.5	4.4
	B. subtilis	21	8.6	86.1		42.4	50.1	3.7
	Act. violaceus-ruber	9	8.6	90.6		20.1	22.3	4.8
	Act. violaceus-ruber	21	9.0	85.7		39.6	45.7	5.8
	Rhizopus sp.	9	8.0	94.9		2.5	3.6	1.5
	Rhizopus sp.	21	8.4	92.9		18.6	19.3	3.9
Fibrin......	Control		8.0	28.8				44.9
	Ps. caudatus	9	8.0	48.3		22.1	33.4	14.1
	Ps. caudatus	39	8.8	47.3		19.8	38.2	8.0
	B. subtilis	9	8.8	58.4		28.7	34.5	9.5
	B. subtilis	39	8.8	45.3		18.6	40.9	6.2
	Act. violaceus-ruber	9	8.6	46.4		12.7	20.6	19.4
	Act. violaceus-ruber	39	9.2	46.5		16.1	35.1	8.2
	Rhizopus sp.	9	8.2	47.4		16.0	25.6	16.6
	Rhizopus sp.	39	8.6	52.1		18.6	29.0	11.2

TABLE 4—*Concluded*

PROTEIN	ORGANISM	AGE OF CULTURE	pH	TOTAL N IN SOLUTION	N RECOVERED AS PROTEIN	NH_3-N		N IN MICROBIAL GROWTH AND IN THE INSOLUBLE PART OF THE PROTEIN
						Found	Corrected	
		days		*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
Albumin....	Control		8.0	58.7	44.2			3.2
	Ps. caudatus	9	8.4	36.5	9.7	15.0	27.1	13.3
	Ps. caudatus	21	8.8	37.2	8.9	15.5	27.3	12.9
	B. subtilis	9	8.4	41.8	10.2	15.0	25.0	10.2
	B. subtilis	21	8.8	41.6	9.3	14.3	25.5	9.1
	Act. violaceus-ruber	9	8.4	56.0	30.2	12.1	14.0	4.0
	Act. violaceus-ruber	21	9.0	54.5	22.2	15.1	18.2	4.2
	Rhizopus sp.	9	8.4	52.9	22.5	11.7	14.9	5.8
	Rhizopus sp.	21	8.4	56.2	14.1	11.1	12.1	3.7
Gliadin.....	Control		7.9	26.5				49.4
	Ps. caudatus	9	8.0	57.6		20.9	29.1	10.1
	Ps. caudatus	39	8.0	59.8		29.5	43.4	2.2
	B. subtilis	9	8.4	27.7		15.2	23.8	39.6
	B. subtilis	39	8.4	26.7		14.1	29.6	33.8
	Act. violaceus-ruber	9	8.6	26.0		10.7	15.6	45.1
	Act. violaceus-ruber	39	9.4	46.5		21.0	38.3	12.1
	Rhizopus sp.	9	8.4	63.7		10.3	15.7	6.9
	Rhizopus sp	39	8.8	64.6		18.8	27.9	6.2
Zein........	Control		8.0	3.0				67.4
	Ps. caudatus	9	8.0	28.4		10.7	10.7	42.2
	Ps. caudatus	39	8.4	61.8		24.6	29.7	3.5
	B. subtilis	9	8.0	7.3		4.1	4.1	62.7
	B. subtilis	39	8.6	12.5		6.4	6.4	58.5
	Act. violaceus-ruber	9	8.2	5.0		1.6	1.6	68.2
	Act. violaceus-ruber	39	8.8	16.2		7.7	7.7	57.4
	Rhizopus sp.	9	8.2	3.8		0.4	0.4	68.4
	Rhizopus sp.	39	8.6	5.7		1.2	1.2	66.2

As in the previous experiments there was in all cases marked increase in alkalinity brought about by the accumulation of ammonia in the cultures. This was responsible for the loss of considerable nitrogen, by volatilization of the ammonia; the longer the period of incubation, the greater was the loss of nitrogen.

The zein was practically insoluble, the gliadin and fibrin somewhat soluble, while the gelatin and casein were quite completely dissolved or dispersed in the solution. The albumin was more soluble in some of the flasks than in others. The decrease in weight of the reprecipitated protein can serve as an index of the decomposition of some of the proteins as casein and albumin. The degree of decomposition of all of the materials can be best compared from the amounts of ammonia produced. The values corrected for the apparent losses of nitrogen by volatilization are most suggestive.

All of the proteins were attacked by all of the organisms used, but not to an equal extent. The gliadin was decomposed practically as readily as any of the animal proteins. On the other hand, the zein was, with most organisms, the most resistant of the proteins studied. But even this material was practically all brought into solution by *Ps. caudatus* at the time of the final examination. These results, however, offer as yet no definite suggestion that plant proteins are alike in their availability to microörganisms or that they differ radically from animal proteins in this respect.

On the basis of the maximum amounts of ammonia produced by the cultures in per cent of total nitrogen, the proteins can be arranged as follows: casein, 63.2; gelatin, 43.4; fibrin, 38.9; albumin, 25.0; gliadin, 38.9; zein, 34.9. Assuming that the losses of nitrogen in the cultures were due largely to volatilization of the nitrogen as ammonia, the above figures will be from 7 to 18 per cent greater. The most important conclusions to be drawn from these experiments is that vegetable proteins decompose as a group as readily as animal proteins and that greater differences may exist between the rates of decomposition of two animal proteins, such as casein and albumin, than between an animal protein (albumin) and a plant protein (gliadin).

The experiments on the decomposition of zein and gliadin were repeated (table 5). The results confirm those obtained in the previous experiment. The bacteria brought about a much greater decomposition of the zein than the fungus or actinomyces, as shown by the reduction in insoluble protein, increase in nitrogen in solution, and ammonia and amino nitrogen formation.

TABLE 5

Decomposition of zein and gliadin by different microörganisms

Incubation, nineteen days

PROTEIN	ORGANISM	pH	WEIGHT OF INSOLUBLE MATERIAL	N IN INSOLUBLE MATERIAL	TOTAL N IN SOLUTION	NH_3-N	NH_2-N
			mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*
Zein	Control	7.2	478	69.2	1.5		
	Ps. caudatus	7.6	115	(15.7)*	63.3	28.2	13.6
	B. subtilis	8.6	255	(32.8)*	33.3	26.3	3.4
	Act. violaceus-ruber	7.6	442	(64.4)*	7.9	4.7	0
	Rhizopus sp.	7.8	442	68.6	6.7	3.4	1.1
Gliadin	Control	7.0	387	57.7	21.0		
	Ps. caudatus	8.4	150	5.2	55.3	22.7	1.1
	B. subtilis	8.4	104	(9.5)*	55.7	33.9	2.3
	Act. violaceus-ruber	8.8	215	(22.6)*	39.1	28.3	1.1
	Rhizopus sp.	8.8	67	4.8	65.2	21.8	3.4

* Determinations lost; values calculated.

The gliadin, however, was decomposed most actively by the fungus. These results further prove that different proteins are attacked at different rates by different microörganisms.

The following experiment deals with the decomposition of four proteins by three active protein-decomposing organisms, representing the groups of fungi, actinomyces and bacteria (table 6). The media, containing 1.0 gram portions of the various proteins in 100 cc., were placed in 250 cc. Erlenmeyer flasks. In order to determine the effects of an available carbohydrate upon the de-

TABLE 6

Decomposition of vegetable and animal proteins by different microörganisms

PROTEIN	ORGANISM	AGE OF CULTURE	DRY WEIGHT OF RESIDUE*	NITROGEN CONTENT OF RESIDUE	NITROGEN CONTENT OF SOLUTION	NH_2-N IN SOLUTION	NH_3-N IN SOLUTION	GLUCOSE USED	GLUCOSE LEFT IN MEDIUM
		days	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*		*mgm.*
Edestin......	Control		978	164.2	2.0	0	Trace	−	0
	Control		984	166.4	1.4	0	Trace	+	795
	Trich. koningi	9	604	85.1	79.6	28.3	32.6	−	0
	Trich. koningi	9	968	120.4	46.8	14.0	25.2	+	Trace
	Act. viridochromogenus	15	862	140.8	25.3	4.5	11.7	−	0
	Act. viridochromogenus	15	823	121.8	41.4	4.8	27.8	+	0
	B. cereus	9	408	65.0	100.3	22.8	40.1	−	0
	B. cereus	9	485	81.0	86.8	30.6	21.8	+	195
Gliadin......	Control		954	135.0	2.1	0	0	−	0
	Control		962	135.6	1.7	0	0	+	795
	Trich. koningi	9	271	28.0	105.5	36.8	32.8	−	0
	Trich. koningi	9	927	90.7	44.8	17.4	10.0	+	75
	Act. viridochromogenus	15	792	109.8	25.1	3.5	15.2	−	0
	B. cereus	9	51	7.3	124.1	36.2	42.6	−	0
	B. cereus	9	559	71.9	62.4	14.0	6.7	+	525
Zein.........	Control		966	144.8	0.6	0	0	−	0
	Control		976	145.9	0.4	0	0	+	795
	Trich. koningi	13	718	97.3	45.5	14.0	26.8	−	0
	Trich. koningi	13	698	67.5	72.7	20.8	36.3	+	0
	B. cereus	13	128	17.9	114.8	27.8	46.5	−	0
	B. cereus	13	853	116.8	17.5	4.2	6.5	+	490
Casein†......	Control				140.2	7.5	1.2	−	0
	Trich. koningi	10	232	17.2	118.0	12.6	44.7	−	0
	Act. viridochromogenus	15	95	10.2	128.6	13.3	19.2	−	0
	B. cereus	9	105	12.3	113.2	38.9	40.9	−	0

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* Residual undecomposed protein and cellular material, not passing through fine filter paper.

† The casein was brought into solution with NaOH. The residual material consists, therefore, almost entirely of cellular material.

composition of the proteins, some of the cultures received 0.8 per cent glucose. The mechanism of decomposition of the proteins was again found to depend upon the nature of the organism, the properties of the protein and the presence of available carbohydrates. The gliadin was most readily attacked by practically all of the organisms. In general, the bacteria were somewhat more active in decomposition than the fungi or actinomyces.

TABLE 7

*Decomposition of proteins and growth of microörganisms**

PROTEIN	ORGANISM	GLUCOSE PRESENT	PROTEIN DECOMPOSED	GROWTH	RATIO: PROTEIN DECOMPOSED / GROWTH
			mgm.	*mgm.*	
Edestin	*Trichoderma*	−	541	167	3.2
	Trichoderma	+	375	359	1.0
	Actinomyces	−	162	46	3.5
	Actinomyces	+	348	187	1.9
	B. cereus	−	622	52	(12.0)?
	B. cereus	+	506	7	(72.3)?
Gliadin	*Trichoderma*	−	854	151	5.7
	Trichoderma	+	495	460	1.1
	Actinomyces	−	240	78	3.1
	B. cereus	−	905	2(?)	(452.5)?
	B. cereus	+	606	203	(3.0)
Zein	*Trichoderma*	−	339	91	3.7
	Trichoderma	+	633	355	1.8
	B. cereus	−	853	15	(56.9)?
	B. cereus	+	276	153	(1.8)?

* Data calculated from table 6.

This decomposition was accomplished by a comparatively small amount of synthesis of cell substance (table 7). At first, considerable quantities of amino nitrogen were found in the cultures, but later most of the nitrogen in solution could be accounted for as ammonia. The sum of the ammonia and amino nitrogen does not equal the amount of the total nitrogen in solution and, even allowing for protein that might be in solution, wide differences exist in many cases. This would seem to indicate that other forms of organic nitrogen than that in α amino groupings were present in large amounts as products of decomposition. After nineteen days' incubation, as much as 39.9 per cent of the nitrogen originally present in the zein medium was recovered as ammonia in 1 case, while the maximum amino nitrogen which was obtained from this protein was only 19.2 per cent. From the gliadin at nineteen days an even smaller proportion of the total nitrogen was recovered in the amino form, although considerable ammonia was found. Up to 43.1 per cent of the initial total nitrogen content was recovered as ammonia and the maximum amino nitrogen was only 4.3 per cent of the total at this time. After nine days, 31.1 per cent of the total nitrogen was recovered as ammonia and nearly as much (26.8 per cent) was found as amino nitrogen. In the case of the edestin, the maximum amounts of protein nitrogen changed to ammonia and amino nitrogen were 24.0 and 18.3 per cent respectively.

Although the amounts of protein left undecomposed were not determined as such, since the protein could not be separated readily from the cellular material, the approximate quantities of protein that were left undecomposed can still be estimated by utilizing a knowledge of the nitrogen content of the protein under consideration and of the cell substance of the organism grown under similar conditions, but in media where the protein was all in solution.

The growth of the microörganisms on the edestin and the decomposition of this protein may be used for the purpose of illustration. The air-dry edestin used in these experiments was found to contain 16.8 per cent nitrogen. The fungus Trichoderma grown on casein media, under exactly similar conditions (as well as on amino acid media), was found to contain about 7.0 per cent nitrogen, when the medium was free from glucose, and about 5.0 per cent nitrogen, when glucose was present in the medium. Let x be the amount of undecomposed protein left in the culture and y the amount of growth produced by the fungus. The following two equations will then hold for the glucose-free cultures:

$$x + y = 604 \text{ mgm.}$$

$$\frac{16.8 \times x}{100} + \frac{7 \times y}{100} = 85.1 \text{ mgm.}$$

By making the proper substitution and solving for x and y, we find that x, or undecomposed protein, is equal to 437 mgm., and y, or synthesized fungus mycelium, to 167 mgm. For the cultures containing glucose:

$$x + y = 968$$

$$\frac{16.8 \times x}{100} + \frac{5 \times y}{100} = 120.4$$

therefore,

$$x = 609 \text{ mgm.}, \ y = 359 \text{ mgm.}$$

These results show that Trichoderma grown in a synthetic medium containing edestin as the only source of carbon and nitrogen, for nine days, decomposed 541 mgm. (978 to 437 mgm.) of the protein in 100 cc. of culture. The nitrogen content of this decomposed protein is equal to 90.9 mgm. $\left(\frac{541 \times 16.8}{10}\right)$; actually there was found in solution 79.6 mgm. of nitrogen. The synthesized mycelium (or 167 mgm., as calculated from the equation) would contain 11.7 mgm. of nitrogen, giving in sum $79.6 + 11.7 = 91.3$ mgm., or very close to the actual amount decomposed. Further, the 79.6 mgm. of nitrogen present in solution might be there in the form of proteoses, peptones, polypeptides, amino acids, ammonia and other soluble nitrogen degradation products. Actually there were found 28.3 mgm. of amino nitrogen and 32.6 mgm. of ammonia nitrogen, leaving 18.7 mgm. of nitrogen in solution unaccounted for. This would tend to indicate that the fungus Trichoderma rapidly breaks down the protein to its lowest derivatives. This has actually been found to be the case, especially if the reaction is not allowed to become too alkaline.

In the presence of the carbohydrate, the edestin was used only as a source of nitrogen and the glucose as a source of carbon, as indicated by the greater amount of protein left undecomposed and the much larger quantity of protoplasm synthesized. The amount of edestin actually decomposed by Trichoderma, in the presence of glucose, was 375 mgms. ($984 - 609 = 375$). The nitrogen content of this decomposed protein would be 63.0 mgm.

$\left(\frac{375 \times 16.8}{100}\right)$; actually there were found in solution 46.8 mgm. of nitrogen. The synthesized mycelium should contain (359 mgm. mycelium and 5 per cent nitrogen) 18.0 mgm. nitrogen. The nitrogen accounted for is thus 64.8 mgm., again quite close to the actual amount of protein decomposed. The solution contains 14.0 mgm. of amino nitrogen and 25.2 mgm. of ammonia nitrogen, leaving only 7.6 mgm. of nitrogen for proline or other nitrogenous substances present in solution and not accounted for by the amino nitrogen figures. This also points to the rapidity and completeness of protein decomposition by this organism.

The actinomyces decomposed less of the edestin than the Trichoderma, even in a longer period of time, as can be readily shown by constructing similar equations and by considering that the nitrogen content of the dry growth of actinomyces (grown on media containing protein) is 8 per cent.

For the non-glucose medium:

$$x + y = 862$$

$$\frac{16.8 \times x}{100} + \frac{8 \times y}{100} = 140.8$$

therefore,

$$x = 816 \text{ mgm.}, \ y = 46 \text{ mgm.}$$

For the glucose medium:

$$x + y = 823$$

$$\frac{16.8 \times x}{100} + \frac{8 \times y}{100} = 121.8$$

therefore,

$$x = 636 \text{ mgm.}, \ y = 187 \text{ mgm.}$$

The bacterium decomposed more of the edestin than either the fungus or actinomyces, both in the absence and presence of the carbohydrate. The dry residual material is composed principally of protein left undecomposed, since a large part of the bacterial cells passed through the filter paper. It is important to point out that, in case of the bacterium, a comparatively larger amount of nitrogen was present in solution than could be accounted for by the amino or ammonia forms. This shows that, although the bacterium decomposed much more of the protein than did the fungus or the actinomyces, a larger part of the nitrogen was left in the medium in the form of certain intermediary protein derivatives.

Similar conclusions can be drawn from the decomposition of the gliadin and zein by the three organisms, as calculated in table 7. *B. cereus* also decomposed more of the gliadin and zein then the Trichoderma.

The activities of these three organisms should not be looked upon as typical of the mechanism of protein decomposition by the three large groups which they represent; other representatives would probably behave somewhat differently.

Economy of utilization of proteins by microörganisms. The data presented in table 7 are calculated values of the amounts of protein decomposed and of microbial growth. The calculated ratios of protein decomposed to microbial cell substance synthesized (growth) indicate that there was much more growth per unit of protein attacked in the presence of glucose. This is explained by the fact that a part of the growth has originated at the expense of the glucose. The values for bacterial development are erratic, due to incomplete recovery of the bacterial cells from the cultures. In general, it appears that the fungus utilized the proteins more economically than the actinomyces since more growth was obtained per unit of protein decomposed.

Further information regarding the economy of utilization of organic matter for the growth of the microörganisms can be derived from the data presented in tables 8 and 9. The relationship between decomposed protein in the medium and microbial protein synthesized is not constant, a fact probably due as much to the difficulties of obtaining absolute measures of growth and protein decomposition as to variability among the organisms. Considering the microörganisms referred to in table 8 in groups, the average ratio for the bacteria is 10.7:1, and for the actinomyces 7.0:1. This would point to more economical utilization of the protein by the actinomyces, a relationship which is in accord with other observations made in this laboratory.

TABLE 8

*Economy of utilization of casein for microbial growth and cell synthesis**

ORGANISM	RATIO: N OF CASEIN DECOMPOSED / N OF GROWTH
No. 1	7.2
B. mycoides	11.1
Ps. caudatus	10.5
B. subtilis	13.4
No. 6	6.0
Sarcina lutea	17.8
B. subtilis	8.8
Act. bobili	5.1
Act. viridochromogenus	5.9
Act. violaceus-ruber	10.0

* Data calculated from table 2.

TABLE 9

*Economy of utilization of casein by microörganisms**

ORGANISM	GROWTH	CASEIN DECOMPOSED	RATIO: CASEIN DECOMPOSED / GROWTH	N IN GROWTH	N IN CASEIN DECOMPOSED	RATIO: N IN CASEIN DECOMPOSED / N IN GROWTH
	mgm.	*mgm.*		*mgm.*	*mgm.*	
Ps. caudatus	15.5	232.5	15.0	1.4	34.8	24.9
	13.5	258.4	19.1	0.1	38.7	387.0?
B. subtilis	5.5	215.5	39.2	0.5	32.3	64.6?
	18.5	277.3	15.0	1.8	41.6	23.1
Act. violaceus-ruber	30.5	195.0	6.4	0.8	29.2	36.5?
	23.5	269.2	11.5	2.0	40.3	20.2
Rhizopus sp.	53.5	188.1	3.5	1.6	28.2	17.6
	35.5	269.2	7.6	2.3	40.3	17.5

* Data calculated from table 4.

Other experiments, not reported here, gave average ratios of protein decomposed to organic nitrogenous compounds synthesized as follows: for bacteria 18.3:1, for actinomyces 11.8:1, and for fungi 10.3:1.

From the data reported in table 9, ratios are calculated both for the quantity of casein decomposed and cell substance synthesized, as well as for the nitrogen content of the casein decomposed and the nitrogen in the growth. Averages of the ratios on the basis of total protein gave the following values: bacteria 22.1:1, actinomyces 9.0:1, fungus 5.6:1. On the basis of nitrogen contents, the most significant values are: bacteria 24.0:1, actinomyces 20.2:1, fungus 17.6:1.

The data from the various experiments, while not consistent as a whole, indicate similar relationships when considered separately. In general, the bacteria required the most protein as a source of energy for each unit of cell substance synthesized; the fungi made the greatest amount of growth per unit of protein decomposed. The actinomyces occupy a position intermediate between the fungi and bacteria.

When considered as a whole, the results of the experiments on the decomposition of plant proteins justify the conclusion that the prevalent idea that vegetable proteins are more or less resistant to decomposition is without foundation. Dr. T. B. Osborne has stated in a personal communication that

> . . . . our experience with the proteins of leaves indicates that these are of a type very different from casein, edestin, or gliadin. These proteins occur chiefly in the colloidal state and when precipitated are to a large extent insoluble in the usual protein solvents. They appear to be combined with non-protein complexes and are rendered soluble in dilute acids or alkalies only after being subjected to hydrolysis. It is a question therefore, whether or not they can be utilized by fungi and bacteria as readily as are the isolated proteins with which you have worked.

The resistance which proteins exhibit to chemical reagents or microbial attack when present in a natural state in plant materials does not justify the opinion that plant proteins as such are not readily decomposable. However, it is quite possible that when proteins are combined with certain nitrogen-free plant constituents they may show much greater resistance to the action of chemicals. Such combinations may also greatly modify their resistance to microbial decomposition. This subject is being studied in detail at the present time in this laboratory and will be reported later.

SUMMARY

1. A study has been made of the rapidity of decomposition of plant proteins by microörganisms, as compared with that of animal proteins.

2. Plant proteins, in a purified condition, when added to a liquid medium containing the necessary mineral salts, were found to be readily decomposed by microörganisms, including bacteria, actinomyces and fungi.

3. There is as much difference between the rates and extent of decomposition of different proteins of animal origin, as between the rates and nature of the decomposition of animal and plant proteins.

4. When the proteins are used as the only sources of carbon for the microörganisms, the reaction of the medium changes rapidly to alkaline and a large part of the nitrogen is lost through volatilization in the form of ammonia.

5. Equations are presented which permit calculations of the amount of protein decomposed and the amount of cell substance synthesized.

6. The ratio of protein decomposed to protein synthesized by microörganisms was widest in the case of the bacteria and narrowest for the fungi; the actinomyces were intermediate.

7. It is suggested that the resistance to microbial decomposition which proteins exhibit when contained in certain plant tissues is due not so much to differences from animal proteins in chemical structure, as to the formation of resistant complexes with non-nitrogenous compounds.

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THE GERMICIDAL ACTIVITY OF VAPORS FROM IRRADIATED OILS

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It has been found that surface smears of various microörganisms are killed when exposed to the atmosphere over certain oils previously treated with ultraviolet light.

A brief survey of the literature indicates some reports on the factors affecting the germicidal power of oils, but only a few papers dealing with emanations of a germicidal nature from oils. This survey also indicates a tendency to ascribe to invisible light the effects caused by any unseen emanations. Thus Reid (1930) explains a germicidal effect of irradiated oil vapor as due to secondary radiations from that oil. We consider that this explanation is not justified, and in this paper present our reasons for a different explanation of the same phenomenon.

LITERATURE REVIEW

Schmidt (1908) thought he observed a radiation effect when he exposed photographic plates to linseed oil and got a screening action by placing a diaphragm of brass, glass or mica between the oil and the plate. Celluloid, paper, gutta-percha and gelatin allowed the fogging material to pass through. Kugelmass and McQuarrie (1924), in work which was later confirmed by Hauxthausen (1925) concluded that the fogging effect of irradiated substances was undoubtedly due to ultraviolet rays emitted from oils. This conclusion has not been substantiated by other investigators and there seems to be almost complete agreement that the fogging effect is due to some volatile chemical compound.

Malowan (1931) found that those oils which contain aldehydes and alcohols rather than esters, are the most active germicidally. There are numerous papers on the germicidal activity of oils, especially essential oils (Gatti and Coyola (1922); Wiechowski (1926); Morel and Rochaix (1927); Anderson and Leitch (1927); Morel, Rochaix, and Sevelinge (1928); Kurgery and Adkisson (1928); Obst (1929)), but very few deal with the effects of the vapors evolved from these oils. Spolverini (1927) has written on the therapeutic value of inhalations of irradiated oils. Rhodes (1925) reported that the successful treatment of tuberculous abscesses with cod liver oil was possibly due to ultraviolet emanations from the oils. Wrenn (1927) exposed bacterial plates to irradiated oil vapors and observed bactericidal action in several cases. He drew the conclusion that this effect was due to light emanations in spite of the fact that he was unable to demonstrate the passage of these emanations through quartz glass. Schöbl and Kusama (1924) placed organisms near oils so that only the volatile constituents at 37°C. and not the oils, came in direct contact with the cultures. They found that the vapors of oils containing acids of the chaulmoogra series have no germicidal power and that the volatile constituents of certain essential oils are highly germicidal to acid-fast bacteria *in vivo*.

PRELIMINARY TESTS

Ten cubic centimeters of Patch's cod liver oil were placed in a Petri dish and irradiated for two hours at a distance of 75 cm. from a 6-inch horizontal quartz-mercury vapor arc. A surface smear of *Staph. aureus* on nutrient agar (Difco) was inverted above this irradiated oil and also above a 10 cc. sample of unirradiated oil and incubated at 37°C. At the end of twenty-four hours there was growth on the agar placed above the unirradiated cod liver oil but there was no growth on that over the irradiated oil. This experiment was repeated with a 4 mm. quartz plate placed between the sample of irradiated oil and the agar plate. There was growth on the agar after incubation. This clearly shows that the bactericidal substance was unable to pass through a quartz plate. It is logical to assume therefore that the substance evolved from cod liver oil is a chemical substance rather than a radiation as has heretofore been assumed.

This fact was demonstrated in still another way. A series of Petri dishes, each smaller than the one preceding it, were placed inside one another alternately inverted. Each stood on glass rods so that there was free air passage from the innermost dish containing the oil to the external inverted dish on which the smeared agar had been placed. After incubation, there was no bacterial growth on this plate. The germicidal substance had traveled from the interior outward over a devious path. The germidical effect, therefore, could not be due to a radiation, for radiations travel in straight lines.

BACTERIOLOGICAL TESTS

A series of oils was tested against *Staph. aureus* to determine how long each had to be irradiated before it became germicidal. All tests were run in duplicate. Five cubic centimeters of each sample were irradiated in the bottom of a Petri dish at a distance of 45 cm. from the arc. One-half cubic centimeter of a 1:100 dilution (750,000) of *Staph. aureus* was placed in the center of each agar plate and the plate tipped to insure complete surface covering. These agar-aureus plates were inverted over the irradiated oils and the two dishes sealed with adhesive tape. After incubation at 37°C. for twenty-four hours the plates were observed for growth. The bottom dish containing the oil was removed from those samples which showed no growth on the agar, replaced by a clean, sterile dish, and the culture again incubated. If there was no growth after twenty-four hours further incubation, the culture was considered killed. If there was growth after the second incubation, the organism was considered inhibited rather than killed.

From table 1 it is apparent that tuna fish, seal and high fatty-acid fish oils are germicidal to *Staph. aureus* without irradiation. Cod, cod liver, elast, L. C. P. fish, linseed, perilla, sardine, walnut, and whale oil give off germicidal vapors only after irradiation of less than two hours. The remaining oils are impotent in this respect even after four hours of irradiation.

A medium that was suitable for the growth of the organism employed was used in all cases. Nutrient broth and nutrient agar were used for *Strep. lactis, Staph. pyogenes-aureus, Sar. lutea, Esch. coli, Eb. typhi, B. subtilis* and *Serratia marcescens.*

TABLE 1

Effect of irradiation time on germicidal power of oil vapors toward Staph. aureus

OIL	TIME OF IRRADIATION OF OIL										
	0 minute	5 minutes	10 minutes	15 minutes	30 minutes	45 minutes	60 minutes	75 minutes	90 minutes	120 minutes	240 minutes
Chaulmoogra	++	++	++	++	++	++	++	++	++	++	++
Cocoanut	++	++	++	++	++	++	++	++	++	++	++
Cod	++	++	++	++	++	−	−	−	−	−	−
Cod liver (Murray)	++	++	++	−	−	−	−	−	−	−	−
Cod liver (Patch)	++	++	++	−	−	−	−	−	−	−	−
Cod liver (Tech)	++	++	++	−	−	−	−	−	−	−	−
Elastoil-D	++	++	++	++	++	++	++	−	−	−	−
Elastoil-R	++	++	++	++	++	++	++	−	−	−	−
Fish (fatty acid)	−	−	−	−	−	−	−	−	−	−	−
Fish (L. C. P.)	++	+	+	−	−	−	−	−	−	−	−
Lime	++	++	++	++	++	++	++	++	++	++	++
Linseed	++	++	++	++	++	++	++	−	−	−	−
Mineral	++	++	++	++	++	++	++	++	++	++	++
Mustardseed	++	++	++	++	++	++	++	++	++	++	++
Oleic acid (U. S. P.)	++	++	++	++	++	++	++	++	++	++	++
Olive	++	++	++	++	++	++	++	++	++	++	++
Orange	++	++	++	++	++	++	++	++	++	++	++
Palmetto	++	++	++	++	++	++	++	++	++	++	++
Palm kernel	++	++	++	++	++	++	++	++	++	++	++
Peanut	++	++	++	++	++	++	++	++	++	++	++
Perilla	++	++	++	++	++	++	++	+	−	−	−
Poppyseed	++	++	++	++	++	++	++	++	++	++	++
Rapeseed	++	++	++	++	++	++	++	++	++	++	++
Sardine	++	+	+	+	−	−	−	−	−	−	−
Seal	−	−	−	−	−	−	−	−	−	−	−
Soyabean	++	++	++	++	++	++	++	++	++	++	++
Sperm (crude)	++	++	++	++	++	++	++	++	++	++	++
Sunflower	++	++	++	++	++	++	++	++	++	++	++
Tuna	−	−	−	−	−	−	−	−	−	−	−
Tung	++	++	++	++	++	++	++	++	++	++	++
Walnut	++	++	++	++	++	++	++	++	++	++	−
Whale	++	+	+	−	−	−	−	−	−	−	−

++, normal growth; +, growth only after the removal of oil; −, no growth.

Glucose broth and glucose agar were employed with *Strep. lactis* and *Cl. sporogenes.* Czapek's media, both broth and agar, were used for *Asp. niger.* Malt broth and malt agar were used with *Sacch. anomolous.*

Each organism was grown at its optimum temperature. *Strep. epidemicus, Strep. lactis, Staph. pyogenes-aureus, Esch. coli, Eb. typhi, B. subtilis* and *Cl. sporogenes* were grown at 37.5°C. *Asp. niger* and *Sacch. anomolous* were incubated at 30°C.

TABLE 2

Effect of oil vapors on typical bacteria and fungi

ORGANISM	TIME OF IRRADIATION OF OIL						
	0 minute	5 minutes	10 minutes	15 minutes	20 minutes	25 minutes	45 minutes
I. Cod liver oil							
Strep. epidemicus	++	++	++	–	–	–	–
Strep. lactis	++	++	++	–	–	–	–
Staph. pyogenes-aureus	++	++	++	–	–	–	–
Sar. lutea	++	++	++	–	–	–	–
Esch. coli	++	++	++	–	–	–	–
Eberthella typhi	++	++	+	–	–	–	–
B. subtilis	++	++	++	+	–	–	–
Cl. sporogenes	++	++	++	++	+	–	–
Serratia marcescens	++	++	++	–	–	–	–
Asp. niger	++	++	++	–	–	–	–
Sacch. anomolous	++	++	+	–	–	–	–

II. Linseed oil

	20 minutes	25 minutes	45 minutes	60 minutes	75 minutes	90 minutes	120 minutes
Strep. epidemicus	++	++	++	++	–	–	–
Strep. lactis	++	++	++	++	–	–	–
Staph. pyogenes-aureus	++	++	++	++	–	–	–
Sar. lutea	++	++	++	++	+	–	–
Esch. coli	++	++	++	++	+	–	–
Eberthella typhi	++	++	++	++	–	–	–
B. subtilis	++	++	++	++	++	+	–
Cl. sporogenes	++	++	++	++	++	+	–
Serratia marcescens	++	++	++	++	–	–	–
Asp. niger	++	++	++	++	–	–	–
Sacch. anomolous	++	++	++	+	–	–	–

III. Perilla oil

	25 minutes	45 minutes	60 minutes	75 minutes	90 minutes	120 minutes	240 minutes
Strep. epidemicus	++	++	++	−	−	−	−
Strep. lactis	++	++	++	−	−	−	−
Staph. pyogenes-aureus	++	++	++	−	−	−	−
Sar. lutea	++	++	++	+	−	−	−
Esch. coli	++	++	++	−	−	−	−
Eberthella typhi	++	++	++	−	−	−	−
B. subtilis	++	++	++	++	+	−	−
Cl. sporogenes	++	++	++	++	+	−	−
Serratia marcescens	++	++	++	−	−	−	−
Asp. niger	++	++	++	−	−	−	−
Sacch. anomolous	++	++	+	−	−	−	−

IV. Oleic acid, U. S. P.

	25 minutes	45 minutes	60 minutes	75 minutes	90 minutes	120 minutes	240 minutes
Strep. epidemicus	++	++	++	++	++	++	++
Strep. lactis	++	++	++	++	++	++	++
Staph. pyogenes-aureus	++	++	++	++	++	++	++
Sar. lutea	++	++	++	++	++	++	++
Esch. coli	++	++	++	++	++	++	++
Eberthella typhi	++	++	++	++	++	++	++
B. subtilis	++	++	++	++	++	++	++
Cl. sporogenes	++	++	++	++	++	++	++
Serratia marcescens	++	++	++	++	++	++	++
Asp. niger	++	++	++	++	++	++	+
Sacch. anomolous	++	++	+	+	+	+	+

++, normal growth; +, growth only after the removal of oil; −, no growth.

Cl. sporogenes was grown "anaerobically" in glucose broth by sealing the tube with sterile vaseline. When exposed to the vapor of the oil it was grown in a Spray culture dish, alkaline pyrogallol being used to produce anaerobiosis. The irradiated oil was placed in a separate dish inside the Spray culture dish.

In a paper which will be published shortly the cause of this germicidal action of irradiated oil vapors will be explained as being very likely due to a peroxidic-oxygen compound and data to prove this contention will be presented.

CONCLUSIONS

1. It has been shown that the vapors of certain oils inhibit or kill bacterial smears on nutrient agar.

2. Certain oils exhibit this property without irradiation with ultraviolet light, whereas others show it only after such treatment.

3. As shown elsewhere this germicidal action has been demonstrated as being due presumably to volatile substances containing peroxidic-oxygen, the formation of which is accelerated by ultraviolet irradiation.

4. *Bacillus subtilis* and *Clostridium sporogenes*, as was expected, were more resistant to this treatment.

5. In general, animal oils are more active in this respect than vegetable oils.

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GROWTH REQUIREMENT OF STAPHYLOCOCCI[1]

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In the course of an investigation on the chemistry of bacterial metabolism it became desirable to develop a simple medium for the cultivation of staphylococci, free from the unidentified and complex compounds present in "extract," "infusion" and "hormone" media. This report describes an attempt to isolate chemically and to identify a substance found essential to "activate" such a medium, thus permitting the growth of staphylococci.

Attempts to develop a synthetic medium, suitable for the growth of all organisms have been made by Uschinsky (1893) and Doryland (1916). Mueller (1922) has studied in detail the chemical requirements for growth of streptococci.

As a metabolic base for our tests the medium of Uschinsky was used, as was also the casein hydrolysate utilized by Mueller. Preliminary tests showed that these two solutions were incapable of supporting growth of freshly isolated staphylococci for more than two generations. In practically all cases there was no growth in the first transfer, provided care was taken to keep the inoculum, and consequently the amount of original medium transferred, very small.

It is generally known that the addition of meat extract to a peptone base medium will permit heavy growth of many organisms, including the staphylococci. Tests showed that meat extract would, in a similar manner, "activate" synthetic media, permitting luxuriant growth of staphylococci. Meat extract alone, when dissolved in saline, proved an adequate medium only in concentration above 0.5 per cent. The presence of 0.01 per cent of extract, however, in the hydrolyzed casein solution, or in

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[1] Experiments conducted while Fellow in Medicine, National Research Council.

Uschinsky's medium promoted heavy growth. The following tests represent a study of some properties of this "activator."

CHEMICAL PROPERTIES

Effect of heat and reaction. Nine flasks containing 100 cc. of 2 per cent meat extract were prepared. In three flasks the reaction was adjusted to pH 7.0; to pH 5.0 in a second set of three; and to pH 9.0 in the remaining three. The flasks were heated in an autoclave at 20 pounds pressure for periods of twenty, forty and sixty minutes, as indicated in table 1. The results given in table 1 show the amount of growth resulting when 1 cc. of the extract solution was added to 5 cc. casein medium, inoculated with *Staphylococcus albus* and incubated. The results indicate that the activator is heat stable at neutrality or in a slightly acid range; but is markedly decomposed by heat in an alkaline solution.

TABLE 1

pH	AMOUNT OF GROWTH		
	20 minutes in autoclave	40 minutes in autoclave	60 minutes in autoclave
5	Good	Good	Slight
7	Heavy	Good	Good
9	Slight	None	None

To test the possibility of an amino acid being concerned in activation a sample of meat extract was subjected to acid hydrolysis. Twenty grams of meat extract were boiled in 200 cc. of 10 per cent sulphuric acid for eighteen hours, under a reflux condenser. The sulphuric acid was removed with barium hydroxide, and the hydrolysate tested for activating power. This property had been entirely lost. Suitable control tests were made to eliminate the possibility of toxic salts of barium remaining in the medium. The probability of an amino acid acting as the activating substance was greatly decreased by this test.

Dialysis. To determine if a non-coagulable protein or other substance of large molecular size was concerned in the activation the ability of the active substance to dialyze was tested. A collodion bag was cast in a 250 cc. Erlenmeyer flask. After washing and removal, 100 cc. of the 2 per cent meat extract solution was introduced into it, and allowed to dialyze for twenty-four hours against distilled water. The dialysate and the residual fluid were found to possess equal amounts of activator, demonstrating its ability to pass through the collodion membrane.

Solubility. The solubility of the active substance in the more common extractive reagents was tested as described below.

Ether. Five cubic centimeters of the 2 per cent extract solution were shaken with 10 cc. ether. On standing, a dark brown viscous layer separated out, as did an intermediate layer of gelatinous consistency. The upper ether layer was siphoned off, and the ether evaporated on a water bath. There was no residual material.

Alcohol. To a 5 cc. portion of 2 per cent extract solution 25 cc. of 95 per cent ethyl alcohol were added. The solution was heated to boiling, filtered, and the precipitate washed with alcohol. Only a very small amount of material was precipitated. The filtrate and washings were combined and evaporated to dryness on the water bath, and the residue taken up in 5 cc. water. Culture tests showed that the filtrate contained the activating substance in amount equal to the original solution, demonstrating its solubility in alcohol.

Benzene. Twenty-five cubic centimeters benzene were added to 5 cc. of the 2 per cent meat extract solution. After agitation the benzene layer was siphoned off, the benzene evaporated on the stream bath, the residue taken up in water and tested. No activating material was present in this residue.

Ethyl acetate. Five cubic centimeters meat extract solution were treated with 25 cc. ethyl acetate, and allowed to stand after shaking. The upper layer was removed, the ethyl acetate removed by evaporation on a water bath, the residue taken up in 5 cc. water, and tested. No active material had passed into the ethyl acetate layer.

Acetone. Five cubic centimeters of the extract solution were treated with 25 cc. acetone. A very bulky, brown precipitate resulted, which could easily be packed by centrifugation, and a clear yellow supernatant decanted off. The acetone was removed from this supernatant by evaporation and the residue taken up in 5 cc. water. Culture tests showed that the original amount of activating substance was contained in the acetone extract.

This test was repeated using 50 cc. extract solution and 500 cc. acetone. The brownish precipitate was washed twice in acetone, then suspended in water and heated until acetone-free. Tests showed it to be entirely free of activating substance, while the filtrate, treated as before, contained the full amount.

These tests show the activating material to be soluble in 80 per cent alcohol and in 90 per cent acetone. Since the bulk of inert material precipitated by acetone is much greater, and since the acetone is more easily removed owing to its greater volatility, use of this chemical seemed desirable for preliminary precipitation. An additional series of tests were made showing that the precipitation of inert material and the extraction of the active material were both equally effective in any concentration of acetone from two to ten volumes. Since further studies on the active substance could be made more easily on concentrated material, 1 pound of meat extract was dissolved in 1000 cc. water, and treated with 3000 cc. acetone. The precipitate was packed by centrifugation, the supernatant decanted, and the acetone-removed from it by heating on the steam bath.

Precipitation. To determine some general chemical properties of the active substance the following series of tests with reagents generally employed in the isolation of bases were made.

Barium hydroxide. Cold saturated barium hydroxide solution was added to 5 cc. of the acetone soluble fraction until precipitation was complete. The precipitate was washed with barium hydroxide, the washings and filtrate combined, made barium-free with sulphuric acid and concentrated on the water bath to 5 cc. Tests showed that all of the active substance was contained in the filtrate.

Silver-barium. To another 5 cc. portion of the acetone-soluble fraction 10 per cent silver nitrate was added until an excess was indicated by the barium hydroxide test. Saturated barium hydroxide solution was then added until the mixture was decidedly alkaline to litmus. The precipitate was filtered off and washed with dilute barium hydroxide. The filtrate and washings were combined and made barium-free with sulphuric acid, then the silver removed with hydrogen sulphide. The filtrate was heated on the steam bath to drive off excess hydrogen sulphide, and concentrated to 5 cc. Culture tests showed it to possess no activity.

A second lot of the acetone-soluble portion was similarly treated. In addition the washed barium-silver precipitate was suspended in water and treated with sulphuric acid and hydrogen sulphide. Culture tests showed that all of the active substance had been precipitated, and that the greater portion of it could be recovered from the precipitate.

Mercuric sulphate. A third 5 cc. portion was treated with an excess of 5 per cent mercuric sulphate dissolved in 5 per cent sulphuric acid. The precipitate was filtered off and washed with 5 per cent sulphuric acid. The filtrate and washings were combined and treated with hydrogen sulphide to remove the mercury. Results of culture tests showed that the active substance had not been precipitated.

Phosphotungstic acid. A fourth 5 cc. portion was treated with an excess of 30 per cent phosphotungstic acid in 5 per cent sulphuric acid. The precipitate was filtered off and washed with 5 per cent sulphuric acid. The filtrate and washings were combined and freed from phosphotungstic and sulphuric acids by the addition of finely powdered barium hydroxide, until the reaction was slightly alkaline, then excess barium was removed by adding sulphuric acid to slight acidity. The precipitates were removed and the filtrate concentrated to 5 cc. Culture tests showed the filtrate to be entirely inactive.

The test was repeated using 25 cc. of the acetone-soluble portion. In addition, the phosphotungstic acid precipitate was suspended in water and treated with barium hydroxide, similarly to the filtrate. All of the active substance had been precipitated and was recovered quantitatively from the precipitate.

These tests show that the active material can be precipitated by barium-silver or by phosphotungstic acid treatment. Since manipulation in the latter process was found to be easier, and also the possibility of loss of the active material by adsorption on metallic sulphides avoided, the remaining bulk of the acetone-soluble portion was treated with phosphotungstic acid and the active substance recovered from the precipitate.

Removal of inert material by crystallization. Preliminary tests showed that the remaining portion of the extract contained large amounts of amino acids and "muscle extractives." Since these substances are not extremely soluble and crystallize well, their removal by fractional crystallization was attempted.

Another pound of meat extract was treated with acetone and phosphotungstic acid as described, and combined with the remainder of the first portion. This solution, slightly acid with sulphuric acid, was heated on the water bath. After considerable concentration a crop of crystals separated out, resembling in appearance half-melted snow, but yellowish in color. These were removed by filtering on a Buchner filter, and recrystallized from water. The second recrystallization yielded colorless crystals. The "mother liquors" were combined and added to the original "mother liquor," and further concentrated. In all, seven such crops of crystals were obtained. Each crop was tested separately and each was found to be free of activating substance after the second recrystallization.

No extensive attempt was made to identify the substances that crystallized out. Carnosine was recognized by its reaction with copper carbonate. Reduction of picric acid indicated the presence of creatinine. Reaction with nitrous acid demonstrated the presence of the amino group, probably contained in dibasic amino acids.

Further concentration. An appreciable bulk of the remaining inert material was removed by treatment with the methods described below.

Mercuric chloride. Preliminary tests showed that an appreciable amount of inactive material could be removed by the use of mercuric chloride, without precipitation of the active substance. Consequently cold, saturated mercuric chloride was added to the remaining solution until precipitation was complete. The precipitate was filtered off, and washed well in mercuric chloride solution. The filtrate was acidified with sulphuric acid and hydrogen sulphide passed through it until free from mercury. The mercuric sulphide was filtered off, the solution neutralized with sodium hydroxide and evaporated to dryness on the water bath and taken up in alcohol, and the alcohol insoluble inorganic salts present were removed by filtration. The solution was again dried on the water bath to remove alcohol and taken up in water. A considerable loss of activating substance occurred during this manipulation, probably due to loss through adsorption on the mercuric sulphide.

Picric acid. Tests made at this point showed appreciable amounts of amino nitrogen to be present. Since this probably represented dibasic amino acids, their removal with picric acid was attempted. Preliminary tests showed that a considerable portion of the remaining material could be precipitated by addition of picric acid, and that this material was inert. To the remaining solution cold saturated picric acid solution was added in excess. A heavy precipitate formed, taking crystalline form on standing over night on ice. This was filtered off and washed with picric acid. The filtrate and washings were combined and made slightly acid with hydrochloric acid, and the picric acid removed by extraction with ether. Tests showed the active substance to be uninjured.

Additional tests. The original amount of meat extract had now been concentrated to a mass of 265 mgm. This material was taken up in 100 cc. of water. This concentrated bulk made practical tests with some rarer chemical reagents.

Gold chloride. To 5 cc. of the concentrate, 10 per cent gold chloride was added in excess. A slight precipitate formed, which was filtered off after standing over night. The precipitate was washed in dilute hydrochloric acid, suspended in water, and treated with hydrogen sulphide until free of gold. The filtrate was similarly rendered free of gold. The volume of each was adjusted to 5 cc. and tests for activating power were made. Neither the filtrate nor precipitate showed any activating property, probably due to loss of the activating substance through adsorption on gold sulphide.

Platinic chloride. To another 5 cc. portion of the concentrate, 5 per cent platinic chloride in 50 per cent alcohol was added. No precipitation resulted, but on the addition of 95 per cent alcohol a fine, granular precipitate formed. This was filtered off, washed in 95 per cent alcohol, dried and taken up in water, then the platinum removed by treatment with hydrogen sulphide. Platinum was removed from the filtrate in a similar manner. On testing, no active material could be demonstrated either in the filtrate or precipitate. Here again, the loss was probably due to adsorption.

Picrolonic acid. To a third 5 cc. portion of the concentrate a saturated solution of picrolonic acid in 95 per cent alcohol was added. A precipitate formed, but was found to consist only of picrolonic acid itself, rendered insoluble by the lowered alcohol concentration.

Electrodialysis. A dialysis cell was built, with parchment membranes approximately 1 inch apart, and adjustable in number up to twelve. Carbon electrodes were used. Dialysis was attempted with varying currents and times, in solutions of high and low conductivity, and of different ranges of reaction. The uniform result was that the activating substance completely disappeared. A possible explanation is that the substance became concentrated in a cell having an alkaline reaction, where, in its more purified and consequently more unprotected form, it was rapidly decomposed.

Nitrogen content. Micro-Kjeldahl nitrogen determinations indicated 17.3 per cent nitrogen. It was observed that the solution would not clear in a digestion mixture containing copper as a catalyst, but cleared rapidly on the addition of hydrogen peroxide. On treatment with nitrous acid no nitrogen was produced. With methyl iodide a characteristic pyridine reaction was obtained. Free pyridine was absent since there was no precipitation with potassium ferrocyanide.

Activating power. Tests for activating property were made, with the results shown in table 2.

TABLE 2

1	5 cc. casein alone	Sterile
2	5 cc. casein plus 0.1 mgm. concentrate	Heavy
3	5 cc. casein plus 0.01 mgm. concentrate	Heavy
4	5 cc. casein plus 0.001 mgm. concentrate	Good
5	5 cc. casein plus 0.0001 mgm. concentrate	Good
6	5 cc. casein plus 0.00001 mgm. concentrate	Fair
7	5 cc. casein plus 0.000001 mgm. concentrate	Trace
8	5 cc. casein plus 0.0000001 mgm. concentrate	Sterile

Using tube 5 as the limit of activity, computation shows that 1 mgm. of the concentrate should be sufficient to activate 50 liters of a synthetic medium.

FORMATION BY BACTERIA

Since certain organisms, most notably the tubercle and colon bacilli, are known to possess the ability to grow profusely in synthetic media, the possibility of their producing a substance with the property of activating synthetic media suggests itself. Borrel, Boez and Coulton (1923) have observed such a substance in media in which tubercle bacilli have been grown.

Two liters of hydrolyzed casein were inoculated with *B. coli-communior* and incubated for four days, the acidity being adjusted twice daily during this period. This procedure yielded a heavy growth of bacilli, which were collected by centrifuging, and washed three times in saline. The supernatant medium was filtered through a Berkefeld "V" filter, and concentrated to 100 cc. on the water bath. Culture tests showed that the medium contained no substance capable of stimulating growth of staphylococci.

The moist, washed bacilli were extracted for five hours with 70 per cent alcohol at 90°, under a reflux condenser. The suspension was filtered, and the filtrate evaporated to dryness on a water bath, then taken up in water. When this bacterial extract was added to hydrolyzed casein, to Uschinsky's medium, or even to the casein hydrolysate used for the growth of the organisms extracted, the medium so treated produced luxuriant growth of staphylococci after inoculation and incubation.

This test showed that these organisms, possessing the ability to grow well in synthetic media, have also the property of synthesizing a substance adequate to stimulate the growth of other species of bacteria. This phenomenon may well explain their ability to grow in a synthetic medium.

The above test was repeated, using ordinary "extract broth" inoculated with staphylococci. The extract of washed staphylococci did not possess the property of stimulating growth of staphylococci in hydrolyzed casein.

To determine whether the substance produced by the colon bacillus was similar in its chemical properties to that occurring in meat extract, the chemical tests done on the active substance in meat extract were repeated, using bacterial extract.

Ten liters of hydrolyzed casein were inoculated with *B. coli* and incubated as before, and the recovered organisms treated in the same manner. The extract was dried, taken up in water, precipitated with acetone, and the acetone-soluble portion treated with phosphotungstic acid, then concentrated by removal of inert material with mercuric chloride and picric acid. The active material from the colon bacilli reacted with all reagents, in a manner identical with that occurring in meat extract.

DISCUSSION

The chemical properties of the active substance contained in meat extract, capable of stimulating the growth of staphylococci, place it in the general chemical class of "natural bases," which class also includes the substances effective in mammalian metabolism, designated collectively as "Vitamine B." These two substances appear to have identical chemical properties as far as they have been determined, as will be evident from examination of the chemical studies of Funk (1922) on the yeast vitamine. Indeed, the property of stimulating bacterial growth has been attributed to "Vitamine B" by Lloyd (1916, 1917), Shiba (1923), Shwartzman (1924–5), and Izrailskii and Runov (1928). Similarly, the ability to produce growth-stimulating vitamines has been claimed for certain bacteria by Pacini and Russel (1918), Sheunert and Schieblich (1923), and by Damon (1924). It is worthy of note that all the organisms for which this ability has been claimed belong in the group growing well on synthetic media.

These results are not considered as indicating a chemical identity between the vitamines promoting mammalian growth and those promoting bacterial growth; however, these two substances appear to belong in the same class of chemical compounds, and to possess in common the property of biological activity in extremely high dilution.

The data secured in these experiments suggest a possible classification of bacteria into three metabolic groups: (1) Organisms possessing the ability to synthesize an activating substance, adequate for themselves and certain other organisms, and by virtue of this property possessing the ability to grow in synthetic media (example, *B. coli*). (2) Organisms requiring an accessory substance in addition to basic energy-producing media, but unable to synthesize it, and growing well in synthetic media if traces of such an accessory substance be supplied (example, staphylococci). (3) Organisms requiring specialized substances for growth, in addition to activators and energy producing substances (example, *B. influenzae* requiring hemoglobin and an additional activator-like substance).

CONCLUSIONS

1. The substance present in meat extract capable of stimulating the growth of staphylococci belongs to the chemical group of

"natural bases." It is heat stable in slightly acid or neutral solutions, but rapidly destroyed in alkaline solution. It dialyzes through collodion membranes.

2. This activating substance is soluble in water, alcohol and acetone, but insoluble in ether, ethyl acetate or benzene. It is precipitated by phosphotungstic acid and silver-barium, but is not precipitated by silver nitrate, mercuric chloride, acidic solution of mercuric sulphate, picric or picrolonic acids. It is apparently strongly adsorbed on metallic sulphides.

3. The activating substance can be concentrated to a degree where less than 1 mgm. is sufficient to activate 50 liters synthetic medium, thus permitting good growth of staphylococci.

4. In chemical and growth promoting properties this substance present in meat extract behaves similarly to a substance produced intracellularly by *B. coli-communior*, an organism that possesses the property of growing profusely in synthetic media. Such a substance is not produced by staphylococci, which lack the ability to grow on synthetic media.

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ERRATUM

In the paper by Professor Z. Morcos on "New Diseases in Young Rabbits" published in the JOURNAL OF BACTERIOLOGY for June, 1932, Vol. 23, pp. 449–454, the word which should have been "tympanites" was unfortunately misspelled "tympanitis."

NEW DISEASE IN YOUNG RABBITS, INFECTIOUS TYMPANITIS

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HISTORY

At a small breeding station near Guiza, young rabbits, between one and five months old, died after a short course of illness.

SYMPTOMS

Native (Egyptian) bred rabbits usually escaped, but foreign breeds, Angora, French, etc., suffered heavily.

Sudden indisposition, appetite not stopped but impaired; diarrhoea was met with in about 50 per cent of the cases, faeces were not too loose, not blood-tinged and not too offensive, clay coloured, but tympanitis was a constant symptom at the very last stages as well as nodding of the head at the very early stages.

The rabbits used to die within 3 days of the appearance of tympanitis and the whole course of the disease took an average of seven days.

POST-MORTEM

Stomach and intestines distended with gas. Caecum impacted with semi-solid clay coloured faeces. Liver highly congested, almost black in colour. The muscles of the body looked slightly darker and no gas was detected on cutting into the muscles. Oesophageal gastric junction inflamed in small patches. These were the constant findings but on rare occasions, peritonitis and ulcers of the small intestines were met with. Post-mortem was made on 12 cases immediately after death and no worms or coccidia were found.

BACTERIOLOGICAL FINDINGS

Smears prepared from the internal organs, muscles and the blood revealed few (one or two bacilli in every 10 or 15 fields) large single (no chains were ever seen) gram + bacilli.

Cultures—aerobically no growth was obtained; anaerobically growth was obtained only in media containing 0.5 per cent agar and no growth was obtained on media containing 1 per cent or more agar. Luxuriant growth was obtained in three days with a fair amount of gas on seeding pieces of internal organs and muscles and on seeding large quantities of blood, (1 or more cubic centimeters). Milk became acid but coagulation was not perfect. Sucrose, glucose, mannitol and lactose were fermented. Serum coagulated media not liquified. Tissue media turned black and the gas produced was inflammable. On serum 0.5 per cent agar slants, small watery isolated colonies appeared after four days incubation.

MORPHOLOGY OF THE ISOLATED ORGANISM

Very actively motile rods, Gram-positive, 3 to 6 microns long, 0.3 to 0.5 micron wide with subterminal spores, sporulation was not frequent and when present, the bacilli looked like racquets. No chains were seen at all on culture or on internal organ smear examination, and growth took place easily under strict anaerobiosis and on all media containing 0.5 per cent or less agar with fairly large amounts of gas production.

ANIMAL INOCULATION

Seven days' old liquid cultures were used, inoculated intramuscularly in the following doses: Rabbits, 0.75 cc.; guinea pigs, 0.5 cc.; pigeons, 0.5 cc.; white rats and desert fawn rats, 0.1 cc. (unless otherwise mentioned).

Experiment 1. Guinea pig 1, rabbit 1, white rat 1, desert rat 1 and pigeon 1 were inoculated and all except the pigeon died between the thirty-sixth and forty-eighth hour of inoculation, the organism was recovered as before described, morphologically and culturally.

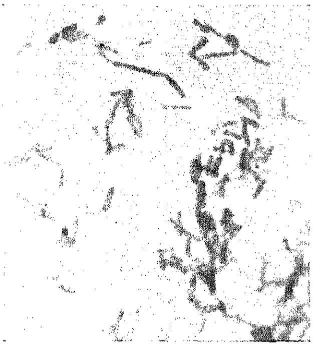

FIG. I

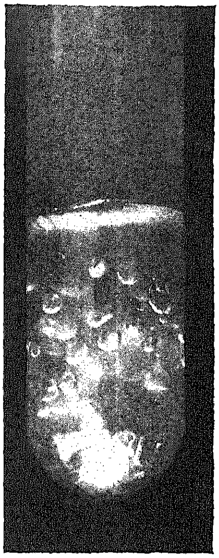

FIG. II

FIG. I. THREE DAYS' OLD CULTURE, CARBOL-FUCHSIN STAIN, OBJECTIVE 1/12, EYEPIECE 10×

FIG. II. SEVEN DAYS' OLD CULTURE, SERUM AGAR AND BROTH, aa, GAS FORMATION

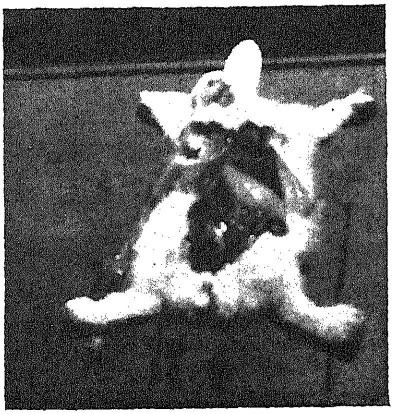

FIG. III. STOMACH DISTENDED WITH GAS, CAECUM IMPACTED

Experiment 2. Guinea pig 2, rabbit 2, white rat 2 and desert rat 2, each received 1/20 cc. culture and all died between the thirty-sixth and forty-eighth hour of inoculation, the organism was recovered from the internal organs and the blood.

Experiment 3. Pigeon 2 inoculated with 0.5 cc. culture; pigeon 3 inoculated with 1 cc. culture; pigeon 4 inoculated with 2 cc. culture; guinea pig 3 (control) inoculated with 0.05 cc. culture; rabbit 3 (control) inoculated with 0.1 cc. culture.

Result. The controls died within forty-eight hours of inoculation and the organism was recovered in pure culture while the pigeons did *not* show any signs of illness and were released after fourteen days.

Experiment 4. Immediately after the death of a naturally infected rabbit, an emulsion of its liver, 1 gram by weight in 30 cc. saline, filtered through 1½ inches diameter Seitz filter. A piece of the same liver was seeded anaerobically and the organism was obtained while the filtrate was seeded and incubated anaerobically with negative result.

Experiment 4. Guinea pig 4 inoculated intramuscularly with 1 cc. filtrate; rabbit 4 inoculated intramuscularly with 1.5 cc. filtrate; rabbit 5 inoculated intravenously with 2 cc. filtrate; guinea pig 5 inoculated intramuscularly with 1/20 cc. culture obtained from the same liver used for filtration (control).

Result. Guinea pig 5 died on the third day of inoculation and the organism was recovered, while those receiving the filtrate did not show any apparent signs of illness and ten days after each received 0.1 cc. culture (the same culture used for guinea pig 5) and proved immune. Hence, no filterable virus could be detected and the filtrate proved protective.

Professor McEwen of the London University, was kind enough to send a typical strain of *B. chauvoei* (isolated from sheep) and immune serum.

Experiment 5. Guinea pig 4, rabbits 4 and 5 (immune to the isolated organism) were inoculated intramuscularly with 0.1 cc. each of the culture of *B. chauvoei*; all died twenty-four hours after inoculation and the organism was recovered in pure culture.

Experiment 6. Guinea pigs 6 and 7, rabbits 6 and 7 each were inoculated subcutaneously with 2 cc. immune Chauveii's serum. Two hours later, each was inoculated intramuscularly with 0.1 cc. culture of the isolated organism, but all the 4 animals were found dead on the morning of the third day of inoculation. Hence there was no relation between the isolated organism in question and the *B. chauvoei*.

IMMUNIZATION

In the following experiments, young rabbits under five months old were used as adults did not suffer under natural conditions.

Experiment 7. Three weeks' old broth culture of the anaerobic organism was passed through a controlled Seitz filter 1½ inches in diameter. Rabbit 8 was inoculated with 0.1 cc. filtrate intramuscularly; Rabbit 9 was inoculated with 0.2 cc. filtrate subcutaneously; rabbit 10 was inoculated with 0.1 cc. filtrate intravenously. After fifteen days, each of these was inoculated with 0.1 cc. live culture from the same culture used for filtration. Rabbit 11 inoculated with 0.1 cc. culture (control).

Result. Rabbit 11 died on the third day of inoculation. The organism was recovered from the dead rabbit, while the rabbits inoculated with the filtrate were kept under observation for five weeks without showing any signs of illness.

Experiment 8. Seven days' old cultures in broth were subjected to 100°C. for five minutes after which ether was added 5 per cent, shaken at intervals for three days and then used. Guinea pigs 8 and 9; each was inoculated with 3 cc. intramuscularly and rabbit 12 inoculated with 3 cc. intramuscularly; all the inoculated animals were kept under observation for ten days when each was inoculated with 0.1 cc. living (controlled) culture but none of the three animals showed any deviation from normality for four weeks following test dose.

Experiment 9. Rabbits 13, 14, 15, 16, 17 and 18 at the breeding farm from which the organism was isolated and which were in contact with recently infected rabbits of the same age, were each inoculated intramuscularly with the heated etherized culture (Experiment 8) dose 1.5 cc., two of the vaccinated rabbits died four days after inoculation and the remaining 4 escaped death and were observed forty days after inoculation.

Experiment 10. Rabbits 19 and 20 were immunized with filtrate of infected liver as in experiment 4 and living culture and their serum secured. Rabbits 21, 22 and 23 at the breeding farm from which the organism was isolated were each given 2 cc. serum on the death of two of the same litter in the same pen, but the 3 rabbits died seven days after the intravenous inoculation of the serum, and the organism was recovered from the dead rabbits.

Experiment 10. A pregnant rabbit, French breed, white, was purchased from the breeding station above referred to and was inoculated with 5 cc. heated ether treated culture, the rabbit gave birth to four after twenty-two days of inoculation and the 4 rabbits are five months old at present and in good condition.

CONCLUSIONS

The outbreak occurred among young European bred rabbits while adult rabbits escaped, and contact guinea pigs at the same farm escaped too.

No filterable virus was demonstrated but filtrate of tissues seems to convey protection.

The organism isolated is similar culturally and morphologically to *B. chauvoei* but it is totally different on cross immunization.

The organism isolated is not pathogenic to pigeons.

The organism is very actively motile, sporulates with difficulty, does *not* form chains, and does not kill laboratory animals before thirty-six hours, it is thus distinguished from *B. welchii*, *B. oedematiens* and *B. oedematis-maligni.*

The organism causes *tympanitis* and death in young rabbits. It may be called *B. tympani-cuniculi.*

Filtrates of infected tissues or cultures are protective to young rabbits.

Heated and 5 per cent ether treated cultures are protective to young rabbits and if given to pregnant rabbits early may produce resistance in offspring.

Serum obtained from immune rabbits does not protect young rabbits.

AGGLUTINATION AND DISSOCIATION STUDIES WITH LACTOBACILLI

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INTRODUCTION

Attempts to classify lactobacilli have been many and varied. Such different criteria of classification as morphology, acid production, carbohydrate fermentation, surface-tension resistance and serological reactions have been used only to result in a tangle of miscellaneous data which tend to increase rather than to clarify the discrepancies in the literature. It is not the purpose of this communication to furnish serological data which will solve the problem, but rather to bring out a few points which may explain some of the apparent inconsistencies in the results obtained by different workers.

Attention has been focussed during recent years upon the phenomenon of dissociation. The lactobacilli have received only scant notice in this field. The agglutination reactions of this group of bacteria when reported, with but few exceptions carry no particular reference to the type of the strain used. Even the sum-total of cross agglutination reactions reported for *L. acidophilus* of intestinal origin, *L. acidophilus* of oral origin and *L. bulgaricus* is very meager.

Kulp and Rettger (1924) abandoned agglutination tests because of spontaneous agglutination. However, they obtained cross complement fixation between *L. bulgaricus* and *L. acidophilus*. Most of the current literature is in agreement to the effect that in all so-called species of lactobacilli whether of *L. bulgaricus* or of *L. acidophilus*, indigenous to the mouth, gastrointestinal tract or soil, the "X" or rough form of colony and the

"Y" or smooth type occur, as well as intermediate forms. The terms "R" and "S" will be used in this paper to indicate the type or phase of the strain in question.

Interest in the phenomenon of dissociation is comparatively recent and this fact must be borne in mind when trying to compare results of workers even during the last decade. Rettger and Horton (1914) considered only the R type as a true acidophilus and the S type as another species. No observation was made at that time that one type might dissociate into the other. Abundant evidence that such is the case is given by a number of writers and our experience will be noted later in this paper. Cruickshank and Berry (1924) recorded agglutination reactions and noted the morphology of the organisms but not of the colonies. As we shall try to show later, the colony type may have considerable bearing upon the cross-reactions between bacterial strains. Howitt (1930) in her comprehensive study of mouth strains noted that the X or R type of intestinal organism did not react with any mouth strains, but that the intestinal Y or S type antiserum agglutinated certain mouth strains. She says that evidently "there is an affiliation between the Y type of stock aciduric rods and those from the mouth in which Y predominated." The oral strains described were all S forms or unstable R forms reverting to S. The R types of intestinal origin cross agglutinated with each other and not with intestinal S forms.

Rosebury, Linton and Buchbinder (1929) noted cross agglutination between most oral strains and intestinal forms of *L. acidophilus.* The dental and intestinal strains used for serum, however, were both from smooth types and the agglutination of only one pure R is recorded. Thomas (1928) reported that *L. bulgaricus* antiserum did not agglutinate other antigens (Döderlein or acidophilus) nor was bulgaricus antigen agglutinated by any other serum. He reported acidophilus antiserum as agglutinating certain Döderlein strains but the colony types used as antigens were not stated. Hilgers (1924) prepared an immune serum to *B. necrodentalis* which agglutinated *B. acidophilus* to a titer lower than that of the homologous antigen but antiacidophilus serum agglutinated *B. necrodentalis* antigen to full titer.

Morishita (1928) found no cross agglutination higher than 1:100 between the mouth strain and *L. acidophilus* of intestinal origin. As pointed out by Hadley, Bunting and Delves (1930) there is an indication that an S type of one strain was compared to the R type of the other. These last mentioned writers prepared a serum against an S type and one against a recently dissociated R variant and obtained almost complete cross-agglutination. As indicated by the work about to be described, the question arises as to whether the R variant they used was a pure or well stabilized R strain.

Hunt and Rettger (1930) used an antiserum for their stock acidophilus (RH) presumably R, though the colony type is not mentioned, and obtained no agglutination with three mouth strain antigens, with *B. bulgaricus* antigen or indeed with any other acidophilus antigen. Ross, Krasnow and Samet (1927) obtained cross agglutination between the Lederle strain of acidophilus and two dental strains, but here again the colony types are not mentioned.

In the present work an attempt was made to use only, as far as could be determined, pure R and pure S strains in the preparation of antisera in order to establish what serological relation exists between these types within a species and between them and other strains of the Lactobacillus group.

SOURCES OF STRAINS

L. acidophilus

S strain—1482-E obtained through the courtesy of the H. K. Mulford Company. In this laboratory this strain had never been found to produce spontaneously any but typical smooth colonies. 1482-G and Y were derived from 1482-E by passage through the human intestinal tract. Ac-41 was kindly sent to us by Dr. W. R. Albus.

R strain—"K"—Kopeloff. This was obtained freshly from an individual ingesting large amounts of milk inoculated with this organism. If in the course of immunization it was found to produce other than typical R colonies a freshly isolated strain was substituted. Derived strains are numbered. Al-34 was obtained together with other strains from Dr. W. L. Kulp. T strains were derived from a culture originally obtained through the kindness of Dr. John C. Torrey of Cornell University. "Scav" was sent to us by Dr. W. L. Kulp.

L. bulgaricus

S strain—B-12 and B-3 was kindly supplied by Dr. W. L. Kulp of the Connecticut Agricultural College, Storrs, Connecticut. This strain had been kept in broth during the course of these experiments and yielded only S colonies.

R strain—B-12. This culture and B-4 obtained more recently than the other from Dr. Kulp had been kept in milk. On plating it was found to contain both R and S colonies. R colonies were fished and the culture maintained as pure R throughout this investigation. B2-US was sent to us by Dr. W. R. Albus.

Oral strains of L. acidophilus

For strains no. 2 and no. 8 we are indebted to Dr. T. Rosebury of the College of Physicians and Surgeons of Columbia University. These strains were isolated from carious teeth. No R strain of oral origin was used for immune serum production since none of those procurable appeared to be pure line strains or were sufficiently well stabilized.

IMMUNIZATION OF ANIMALS

Duplicate rabbits were inoculated intraperitoneally three times a week with saline suspensions of living organisms obtained from a washed forty-eight-hour surface growth upon casein-digest lactose-agar. Plates were streaked at the same time that the Blake bottles were inoculated and examined for purity of colony-type. This was very necessary in the case of the R acidophilus strain for occasionally the colonies were found to dissociate. Once or twice during the period of immunization a freshly isolated K strain had to replace the one in use. The suspensions used in all instances were made up in saline to a density of No. 4 on the McFarland nephelometer scale. This approximated about two billion organisms per cubic centimeter. Doses increasing from 0.2 to 1.0 or 2.0 cc. were used, depending upon the loss of weight of the animals. From 12 to 16 inoculations were given in all and the rabbits bled six to eight days after the last injection. Titers with homologous antigens of 1:1280 to 1:5120 were obtained for each of the 5 types used.

AGGLUTINATION AND ABSORPTION METHODS

Agglutination tests were run macroscopically in the usual way. The antigens were made up to a nephelometer density of 4 and stored in the ice-box either in this density or in heavy stock suspensions to be diluted when desired. Where spontaneous clumping occurred in the R strains tested the organisms were suspended in distilled water and the serum dilutions were made up in half-strength physiological saline making a final dilution of 0.21 per cent saline in the tubes. Sera were diluted in series to make final dilutions of from 1:20 to 1:10,240 and saline controls were always run. The total volume of diluted serum plus antigen was 0.4 cc. in 7.5 by 1.0 cm. tubes. These were incubated for two hours at 40 to 45°C. and placed in the ice-box over night.

In spite of the acknowledged importance of agglutinin-absorption tests as checks upon the rough agglutination procedure it is surprising that such methods in connection with the lactobacilli have been rarely used. McIntosh, James and Lazarus-Barlow (1924) reported that most of their oral strains absorbed the agglutinins of their two type sera but that *B. bulgaricus* showed no absorption. Morishita (1929) mentioned agglutinin-absorption tests in connection with dental organisms but published no data. We believed that reciprocal absorption experiments might yield valuable information and the following procedure was therefore carried out.

Washings from casein-digest agar surface growths or bacterial sediments from casein-digest broth were centrifuged, after filtering through glass wool to remove agar or other extraneous material, until the bacteria were packed as heavily as possible. The absorption dose was determined for each serum by emulsifying the sediment in different proportions in diluted homologous serum and incubating with occasional shaking at 45°C. for from four to five hours. The tubes were then placed in the ice-box over night. In all but the bulgaricus S sera the absorbing dose was found to be one part of packed bacteria to 9 parts of serum diluted 1:20. With the S serum mentioned a dilution of 1:40 was used suspending the antigen 1:5. This serum had a higher agglutinating titer than the others.

These absorbing doses, or some slightly heavier, were used with the heterologous antigens to be tested and the sera were absorbed only once by the bacteria. Repeated absorption was not considered necessary as the criterion of 5 per cent or less of the final titer for unabsorbed sera as postulated by Krumwiede, Cooper and Provost (1925) was obtained after absorbing with homologous antigens. Agglutination tests with unabsorbed sera incubated with the absorbing sera were always made at the same time. Whenever R antigens were to be tested necessitating dilute saline the serum used for absorption was diluted in distilled water instead of 0.85 per cent saline.

AGGLUTINATION AND ABSORPTION RESULTS

In table 1 are recorded the titers of the agglutination reactions of the five type sera with a number of strains of *L. acidophilus*, *L. bulgaricus* and two dental strains obtained from Dr. T. Rosebury. The numbers and letters indicating these strains will be familiar to laboratory investigators who have worked with them. Briefly, these titers show that serologically smooth strains of *L. acidophilus* are more closely related to smooth strains of *L. bulgaricus* than they are to rough strains of *L. acidophilus*. The same relationship is observed with rough variants. The table also shows that the "roughening" of the S type of colony, designated here as "O" or intermediate, does not necessarily imply an antigenic difference. Certain aspects of the dissociation phenomenon will be discussed later on in this paper.

Tests were made on the heat-stability of the type antigens. Heating in boiling water for one hour did not alter the agglutinability of any type antigens used in the preparation of immune sera. In the case of R antigens the spontaneous agglutinability was increased so as to make the specific agglutination with homologous sera difficult to read. So far as determined, then, these antigens contain no heat-labile fraction.

In table 2 the results of reciprocal agglutinin-absorption tests are shown. Again it will be seen here that the pure S antigens of the acidophilus and bulgaricus strains absorb agglutinins from all the S sera but not from any R sera. A similar absorbing specificity occurs with R antigens.

TABLE 1

Agglutination reactions of lactobacilli; L. acidophilus strains (intestinal)

STRAIN	COLONY TYPE	ANTISERA				
		L. acidophilus S	*L. acidophilus* R	*L. bulgaricus* S	*L. bulgaricus* R	Oral no. 2
1482-E	S	2,560	0	2,560	0	0
1482-G	R; O	640	0	1,280	0	
Y-2	S	640	0	2,560	0	
T (1)	O	2,560	0	2,560	0	
T (2)	O	5,120	20	5,120	0	
K (1)	R	20	2,560	0		0
K (2)	R	20	2,560	0		
K (3)	R	40	2,560	0	2,560	
Al-34	R	0	2,560	0	2,560	
Ac-41	S	640	0	1,280	0	
Scav	O	2,560	0	2,560	0	
L. bulgaricus strains						
B-12 (1)	S	1,280	0	1,280	0	0
B-12 (2)	R	20	2,560	0	2,560	
B-12 (3)	S	2,560	0	10,240	0	
B-3	O; S	2,560	0	2,560	0	
B-4	R	0	2,560	0	1,280	
B2-US	R	20	2,560	0	2,560	
Oral strains						
2	S	2,560	160	5,120	80	2,560
8	S; O	160				2,560

R, rough; S, smooth; O, intermediate.

It is to be regretted that we have so few data on oral strains. It was very difficult to secure suitable ones. Many, though

TABLE 2

Agglutinin absorption tests

ANTIGENS	COLONY TYPE	AGGLUTINATION TITER WITH ANTISERA FOR				
		1482-E S Acidophilus	No. 2 S Oral	B-12 S S Bulgaricus	B-12 R R Bulgaricus	K R Acidophilus
Unabsorbed sera						
1482-E	S	5,120	0	5,120	0	0
B-12	S	2,560	0	5,120	0	0
B-12	R	20		0	640	1,280
K	R	20	0	0	1,280	2,560
Oral No. 2	S	2,560	2,560	5,120	80	160
Oral No. 8	S; O	160	2,560			
T-1	O	5,120		5,120		
Sera absorbed with 1482-E (S)						
1482-E	S	0	0	0		0
B-12	S	0	0	0		
K	R					1,280
Oral No. 2	S	40	1,280			
T-1	O	0				
Sera absorbed with B-12 (S)						
1482-E	S	0		0		
B-12	S	0	0	0	0	
B-12	R				2,560	
Oral No. 2	S		1,280	0		
Sera absorbed with K (R)						
1482-E	S	2,560				
B-12	S	1,280*				
B-12	R					0
K	R					0
Oral No. 2	S	1,280				
Sera absorbed with B-12 (R)						
B-12	S			2,560		
B-12	R					0
K	R					0

* In this test the titer of the unabsorbed control was 1,280.

TABLE 2—*Concluded*

ANTIGENS	COLONY TYPE	AGGLUTINATION TITER WITH ANTISERA FOR				
		1482-E S Acidophilus	No. 2 S Oral	B-12 S S Bulgaricus	B-12 R R Bulgaricus	K R Acidophilus
Sera absorbed with oral No. 2 (S)						
1482-E	S	5,120	0	5,120		
B-12	S	2,560	0	2,560		
Oral No. 2	S	0	0	0		
Sera absorbed with oral No. 8 (S)						
1482-E	S	2,560				
B-12	S	320†	2,560			
Oral No. 2	S	0	2,560			
Oral No. 8	S	0	0			

† In this test the titer of the unabsorbed control was 320.

smooth in colony appearance, agglutinated spontaneously and no satisfactory method was found to make stable suspensions. With recently isolated strains from the mouth, moreover, it is difficult to obtain definite agglutination and the same is true of "native" strains isolated from the intestinal tract. The satisfactory results with stock laboratory strains are no doubt due to their prolonged cultivation upon artificial media. Many of the available oral strains were only recently isolated. In the agglutination results with the oral antigens and sera it will be seen that oral strain no. 2 agglutinates to titer with both smooth intestinal sera but that it removes by absorption only its *own* agglutinins. The smooth antigens do not agglutinate with oral smooth serum nor do they absorb the antigen no. 2. Another oral strain no. 8 absorbs no. 2 agglutinins from acidophilus serum but not from no. 2 homologous serum. These few findings appear to corroborate the statement of Rosebury, Linton and Buchbinder (1929) that "on the whole there is indication of a slightly greater homogeneity in the intestinal group than in the dental group."

The type of agglutination with smooth antigens was usually definite and clear cut. Granular clumps settled to the bottom of the tube, the supernatant remaining clear. Agglutination of rough antigens was frequently difficult to read even with a stable suspension. The tubes appeared muddy and the particles which were often easily broken up on shaking were usually studied with a hand-lens. Heavy flocculation as described by some authors in S antigens was not observed.

DISSOCIATION STUDIES

It is beyond the scope of this paper to essay a complete discussion of the dissociation phenomena occurring among these lactobacilli but a few fragmentary observations have been made and the antigenic character of dissociated types studied.

As has been noted by others, the most stable colony type here is that characterized as "smooth." Contrary to what usually occurs in other bacterial species the common spontaneous dissociation here is from R to S. Cultures of the R type of acidophilus grown in milk, casein-digest agar or broth show a tendency to change to S. A striking example of such a dissociation, involving a complete antigenic change to S, occurred during the preparation of cultures for rabbit immunization with the K (R) strain. This culture was isolated from an individual fed acidophilus milk prepared in this laboratory and was acclimatized to broth about August 15, 1930. An agglutination test on September 11 showed a titer in R serum of 1:2560 and S serum 1:20. This culture was transplanted every other day on agar for the preparation of antigens and examined carefully for purity of type. The culture on September 26 showed some colonies with smooth characteristics as well as R colonies and that on September 29, 100 per cent S colonies. Agglutination tests on these cultures were as follows:

DATE	R SERUM	S SERUM	COLONY TYPE
September 11	1:2,560	1:20	Pure R
September 26	0	1:80	Intermediate, some R
September 29	0	1:2,560	100 per cent S

Cultures between September 11 and 26 showed pure R colonies but were not tested for agglutination. The above data indicate a rapid and complete change in antigenic character in 3 transplants. The resulting S culture also showed the same absorbing power as the type S culture (1482-E) and has remained pure S. Attempts to dissociate it back to R have not been successful.

The change from S to R in the intestinal types has not occurred spontaneously with us in well stabilized strains. An indication that this might occur in a recently dissociated strain was illustrated by the behavior of the culture "Torrey." This culture when first tested showed intermediate colony types but it was agglutinated to high titer by S sera only. A stock culture on agar about fourteen weeks old, when transplanted to fresh agar gave 100 per cent typical rough colonies and was agglutinated by R serum only.

Attempts to force dissociation have met with only moderate success. Cultures of S acidophilus in 5 and 300 cc. amounts of casein-digest broth containing lactose were kept incubated at 37°C. as long as twenty-three days but no R colonies were observed. A similar experiment was carried out with a culture of R acidophilus. In 5 cc. amounts the "intermediate" and "smooth" forms predominated after a week and continued doing so for twenty-five days when the experiment was discontinued. In the 300 cc. flasks there appeared to be a fluctuation, as though the apparently S forms had reverted back to the R during the course of growth. A similar fluctuation was noted in a 5 cc. broth culture containing 10 per cent ascitic fluid. Here, 50 per cent dissociation to an intermediate type was noted on the second day of incubation but the percentage diminished to 3 per cent O colonies on the third day. By the sixth day it was again 50 per cent. Then the numbers of intermediate colonies diminished until the eighteenth day when only R colonies were observed. The culture was sterile on the twenty-first day.

Again, dissociation from R to S may be true only morphologically. A two weeks' old casein-digest broth culture of pure R type showed some apparently smooth colonies which were isolated. Rapid serial transfers resulted in a complete reversion to the R type in 5 or 6 transfers. This reversion does not occur with stabilized S types. An immune serum was prepared from this apparently S strain which agglutinated its antigen at 1:640, though the agglutination was indistinct. R strains of *L. bulgaricus* and *L. acidophilus* agglutinated with this serum at 1:640 but with S types only in a dilution of 1:40 or 1:80. After several transplants on agar the culture gave both R and S colonies. The serum used above agglutinated both these strains to titer but an acidophilus S serum agglutinated the new S strain in only 1:320 dilution, whereas the R serum agglutinated both smooth and rough antigens to titer. Table 3 shows these results more clearly. From this experiment it would seem that a variant with apparently smooth morphology has attained only slightly a smooth antigenic character while losing none of the original R properties except that of morphology.

TABLE 3

Agglutination reactions of L. acidophilus, L. bulgaricus type strains and a strain (SK) morphologically S dissociated from an R acidophilus strain

STRAIN	ANTISERA		
	SK	*L. acidophilus* R	*L. acidophilus* S
L. acidophilus R	640	2,560	20
L. bulgaricus R	640	2,560	0
L. acidophilus S	40	0	2,560
L. bulgaricus S	80	0	2,560
SK (S)	640	2,560	0
R colony from SK (S)	640	1,280	0
S colony from SK (S)	640	2,560	320

Dissociation from S to R was attempted by serial transfers of the acidophilus strain 1482-E (S) in 10 per cent immune serum added to lactose-free casein-digest broth of pH 7.5. Lactose was omitted as it was thought acid formation might inhibit dissociation as suggested by Dulaney (1928). Control transfers were made in similar tubes using 10 per cent normal rabbit serum. Two cubic centimeter amounts of broth were used and about 0.1 cc. transferred every twenty-four or forty-eight hours in inoculation. Colonies on casein-digest lactose agar grown from each transfer tube were examined. Marked agglutinative growth occurred in S serum but not in the normal rabbit serum broth throughout the experiment. In all, 40 serial transfers were made. During the first 19 the only difference in colonies appeared to be a slight roughening on the edges of the colonies developing from the S serum broth. The agglutination properties of cultures grown on agar inoculated from serial transplants numbers 19, 25, 30 and 40 from both S serum and normal serum broths were tested. These reactions are noted in table 4. Colonies from transplants 27 to 30 from S serum broth showed small projections from the edges. A filamentous outgrowth from a colony from no. 28 was fished and isolated. Colonies from transplant no. 40 in S serum were indistinguishable from those in the control normal serum broth.

TABLE 4

Agglutination reactions obtained from different serial transfers of 2 pure S strains of L. acidophilus in 10 per cent S immune serum broth and 10 per cent normal rabbit serum broth

SERIAL TRANSFER NUMBER		ANTIGENS FROM CULTURE IN 10 PER CENT S SERUM		ANTIGENS FROM CULTURE IN 10 PER CENT NORMAL RABBIT SERUM	
		1	2	3	4
		R serum	S serum	R serum	S serum
19	Strain 1	80	160	0	160
	Strain 2	80	40	40 (?)	80
25	Strain 1	640	640 (?)	0	80
	Strain 2	80	160	0	80
30	Strain 1	320	160	0	320
	Strain 2	160	160	20	160
40	Strain 1	40	640	0	640
	Strain 2	20	320	20	320

A similar set of serial transfers and agglutination tests were carried out at the same intervals using the S acidophilus culture spontaneously dissociated from R which was described earlier in this paper. Little if any change in morphology of the colonies was noted in this experiment but the agglutination tests gave results very similar to those when using 1482-E, that is, a temporary acquisition of R antigenic property (see columns 1 and 3). At the end of the series a slight reduction in titer occurs with S serum, but a similar phenomenon is observed in normal serum (see columns 2 and 4). These values had returned to practically the same as those of the culture before culturing in the serum broths.

The point at which the R properties were most marked in the strain isolated from transfer no. 28, just mentioned, deserves description. The colonies were rougher than any observed in the dissociation of 1482-E though they were not of the extremely filamentous R type seen in R cultures. They had very irregular edges, a granular appearance and contained no definite dark center. In broth a heavy mat of growth settled out on the bottom of the tube which is quite characteristic of R organisms and the Gram stains showed typical heavy Gram-positive filaments. A great deal of trouble was experienced in preparing a suitable antigen as marked spontaneous flocculation occurred even in distilled water. Numerous attempts were made to remove this obstacle, the most successful of which was alcohol and chloroform extraction as described by White (1928). The antigen made by this method some months after the isolation of this variant showed agglutination with S serum in 1:2560 and with R in 1:160. An absorption test using untreated antigen with S serum (1482-E) showed almost but not quite as complete absorption as with the homologous antigen. Here again is another example of an apparently dissociated strain which still carries considerable antigenic property of the original type while acquiring many characters of the dissociate. Attempts were made to make serial transplants of R acidophilus in 10 per cent R serum but the cultures grew poorly and died out after one or two transfers.

DISCUSSION

According to these studies it appears that if *L. acidophilus* and *L. bulgaricus* are separate species, a distinction cannot be drawn on the basis of agglutination reactions. Smooth strains of one are serologically identical with smooth strains of the other and similar results occur with rough strains. There is no doubt that under suitable conditions one phase can pass to the other with corresponding changes in colony type and serology. While such striking similarity between the oral strains of *L. acidophilus* studied and the intestinal strains of *L. acidophilus* was not demonstrated it is quite evident that the relationship is a very close one and it is not at all improbable that native intestinal strains might be found that would correspond no more closely to the type intestinal strain than do these mouth forms. However, as Hadley (1927) p. 159 points out "antigenic relationship between the S, the O, and the R forms arising from the same pure line may often be less marked than the relation shown between some of these types and unrelated bacterial species when judged by agglutination, precipitation or complement-fixation tests and results." Whether a distinction should be made on the basis of the ability to become implanted in the intestinal tract could be determined only by carefully controlled experiments using separately R and S forms of each so-called species.

According to the comprehensive review of Hadley, it seems evident that in this study we are dealing really with the "O" and "R" heat-stable forms described by Weil and Felix, Goyle and others. True "S" (H), the heat-labile antigen they described, was not encountered though it may exist. So far as we can ascertain, this aspect of the subject has not been studied with reference to the lactobacilli. In agreement with Hadley (1927), p. 150, we find the O and R antigens in apparent purity although in strains undergoing dissociation or in an unstable state combinations of R and O may be observed. Arkwright (1931) says, "The R somatic substance does not react either in agglutination, absorption, or as agglutinogen in the same way as S somatic antigen, but evokes in the rabbit R somatic agglutinins which agglutinate the R form, and not the S, and can be absorbed by the R form only." Burnet (1930) holds the same view: "When suitable strains are used it can be shown that the S and R antigens are quite distinct, giving no cross agglutination and absorbing specifically the corresponding agglutinins."

The fact that several authors, among them, Soule (1928), Krumwiede, Cooper and Provost (1925), Hadley, Bunting and Delves (1930) and others report partial or entire cross agglutinative reactions between R and S forms might be explained on the basis that they were not dealing with pure line strains. In fact Hadley and her coworkers report almost complete cross agglutination between a smooth strain and a so-called rough strain dissociated from it. In our opinion the R strain colony as she illustrates it resembles closely what we have called an intermediate type and which is usually S serologically.

Instances of colony morphology of one dissociate, having serological characteristics of the other as described by us in attempts to force dissociation may be found in the literature. Arkwright and Pitt (1929) mention smooth colonies obtained by growing R cultures of the typhoid group in R serum which show an "ambiguous" character. Burnet (1929) obtained "smooth-looking" colonies by treating an R culture of *B. enteriditis* with rough phage but they remained R antigenically; while others he found acquired S serological properties. Many of the dissociation experiments described giving the degree of dissociation as determined by the percentages of typical R and S colonies may indicate only a partial dissociation if antigenic character as well as colony morphology is considered.

Our evidence of the possibility of reversion back to the original type as described in the cultures maintained for a period of time in broth and in rapid serial transfers corroborates the findings of de Kruif and Morishima as cited by Hadley (1927), p. 218. We went a step farther in our studies of serial transplants by testing the changes in agglutinative properties not only by the homologous (S) immune and normal rabbit sera but also by the (R) serum homologous to the dissociate.

SUMMARY

1. Pure line rough and smooth strains of *L. acidophilus* appear to be antigenically distinct. The same holds true for rough and smooth strains of *L. bulgaricus.* However, the rough forms of *L. acidophilus* and *L. bulgaricus* are serologically indistinguishable. The same is true of smooth forms of these two species. Oral

strains are serologically closely related to intestinal strains of *L. acidophilus*.

2. No heat-labile fraction was found in either S or R antigens of these lactobacilli.

3. Incompletely dissociated or unstable strains may exhibit a morphology which does not correspond to their antigenic character or they may retain antigenic properties characteristic of both R and S.

4. Complete dissociation from R to S occurs spontaneously but it is difficult to obtain antigenically pure R forms from S.

5. Partially dissociated types in cultures under continuous incubation and in rapid serial transfers tend to revert to the original type.

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THE INTERPRETATION OF DIRECT DIFFERENTIAL COUNTS OF COLON-AEROGENES ORGANISMS IN WELL WATERS[1]

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Recently developed differential plating methods of enumerating colon-aerogenes organisms are in need of further study from the standpoint of interpretation of findings. Such a method, originally devised by Noble (1928) and later revised and simplified for use in routine water analyses (Tonney and Noble (1931a), has been used by us in studying the sanitary significance of *Bact. coli* and *Bact. aerogenes* in water supplies (Tonney and Noble (1931b)), and also for studying the relative persistence of the two types of organisms in nature (Tonney and Noble (1931c)). The data of these studies seem to indicate that the *Bact. coli* count in this medium has a much closer and more consistent relationship to fecal sources of pollution than has the *Bact. aerogenes* count.

In addition to yielding separate counts of the *Bact. coli* and *Bact. aerogenes* types, as well as the total group count, the differential plating method possesses the inherent advantages of simplicity, accuracy, economy of time, and relative freedom from the complications of symbiosis, which recommend it for more general use.

Being impressed with the promise of the plating method in water analysis, and recognizing its need of evaluation in the light of the accepted standards based on the older fermentative methods, the authors undertook a comparative study of the findings of the two methods as applied to water samples and their correlation with a sanitary survey of the sources of fecal pollution. Private well waters were selected as best adapted to the purpose, because of their wide variation in sanitary quality.

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[1] Read before the laboratory section of the American Public Health Association in its sixtieth Annual Convention at Montreal, B.C., September 14, 1931.

Accordingly, 147 well waters, collected from rural districts adjacent to Chicago, were examined during the summers of 1930 and 1931. The colon-aerogenes content was determined in parallel, by direct plating in ferrocyanide-citrate-agar, and by inoculation of fractional portions into lactose broth and subsequent confirmation, by the standard method of water analysis (American Public Health Association, 1925).

The sanitary survey was made by members of the laboratory staff and was directed primarily toward discovery or reasonable exclusion of definite sources of fecal pollution in the environment.

In all instances where inconsistencies occurred in the findings of the two bacteriological methods, "follow-up" samples were collected personally by the bacteriologist and the sanitary survey confirmed. The collection of data was continued until a definite judgment of the safety of the water was formed. Often new factors were discovered in these secondary investigations, which explained apparent discrepancies in the earlier data. In the final sanitary judgment, the wells were classified as (1) satisfactory, (2) doubtful, and (3) unsatisfactory, on the basis of the sanitary survey alone. The bacteriological findings were then correlated with the sanitary judgment in such a way as to show the relation of the final sanitary judgment to:

1. The separate *Bact. coli* count.
2. The separate *Bact. aerogenes* count.
3. The colon-aerogenes index, determined by the standard method of water analysis.
4. The number of extraneous organisms (rough estimate) appearing on the differential plates.

The data are given in figure 1.

To bring out the relationships, the data are arranged as follows, reading from above downward on the chart:

First, in the order of satisfactory, doubtful and unsatisfactory surveys when all bacteriological findings were negative (column 1).

Next, in the order of the colon-aerogenes index by the standard

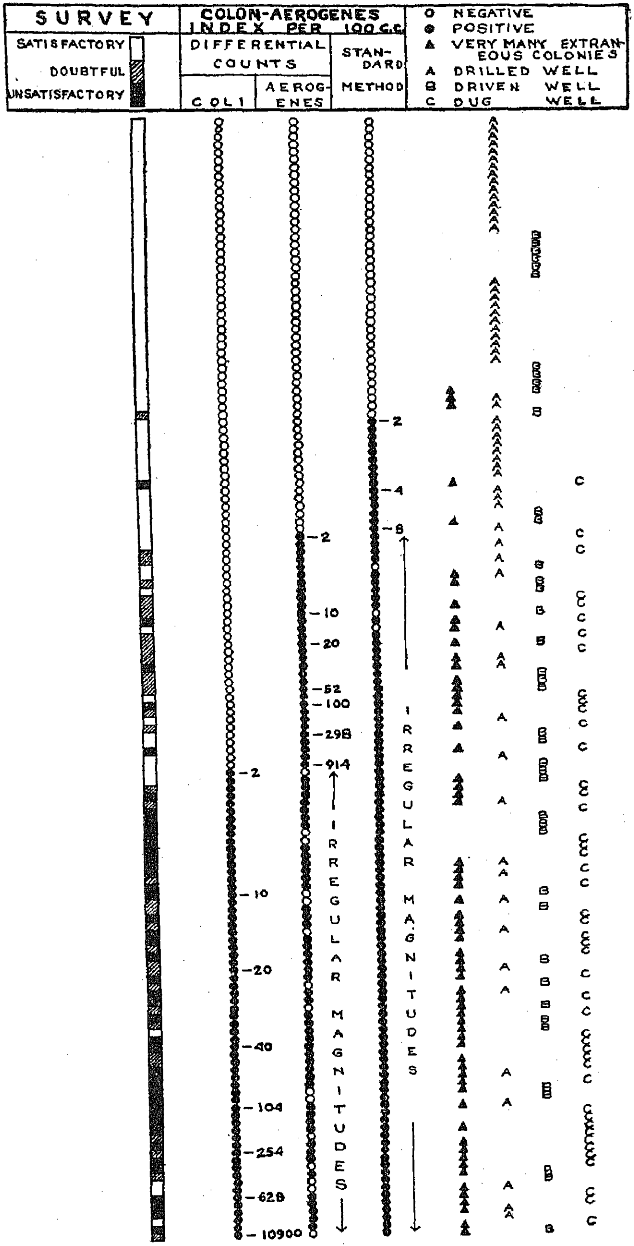

FIG. 1. RELATION OF COLON-AEROGENES FINDINGS TO THE SANITARY SURVEY

method, when the direct differential count of both organisms was negative (column 4).

Next, in the order of the direct *Bact. aerogenes* count, when the *Bact. coli* count was negative (column 3).

And finally, in the order of the direct *Bact. coli* count, irrespective of all other findings (column 2).

On this basis, some very interesting relationships develop.

The clearly satisfactory sanitary surveys, viz.: those in which no source of fecal pollution of the water could be found by repeated inspection, group themselves quite definitely with the negative bacteriological findings by the direct plating method and also in general with the negative findings by the standard method. However, 26.9 per cent of these satisfactory surveys were associated with positive colon-aerogenes findings by the standard method alone. These indices were of low magnitudes (2 to 8 per 100 cc).

A number of doubtful sanitary surveys group themselves with positive *Bact. aerogenes* and negative *Bact. coli* counts by the differential plating method, with the standard method indices usually positive.

The clearly unsatisfactory and the remaining doubtful surveys fall en masse with the direct *Bact. coli* count.

There are interesting sidelights also in the relationships. Thus:

The plates showing numerous extraneous organisms (column 5) which may be considered generally comparable to high bacterial counts in plain agar, are almost without exception identical with the positive *Bact. aerogenes* counts.

The drilled wells (column 6) are grouped primarily with the satisfactory sanitary surveys.

The driven wells (column 7) are fairly well scattered, with a tendency to concentrate in the doubtful and unsatisfactory ranges rather than in the satisfactory.

The dug wells (column 8) fall almost exclusively in the doubtful and unsatisfactory ranges.

From the standpoint of interpretation, the points that stand out in analysis of these data are:

Of the bacterial criteria, the separate *Bact. coli* count seems to be most consistently associated with the presence of definite sources of fecal pollution. It appears to be the surest and most precise indication of dangerous fecal pollution at present available.

The *Bact. aerogenes* count alone, in the absence of *Bact. coli* in the differential plates, appears to be rather an indication of potential danger of pollution than of actually impending fecal pollution. Its significance seems about parallel to that of a high miscellaneous bacterial content as shown by extraneous forms in the differential plates.

The standard method procedure is found in these studies to be sensitive to both the unsatisfactory and doubtful ranges of sanitary surveys, but is also positive in 26.9 per cent of the wells classed as satisfactory after repeated sanitary surveys. In other words, it is apparently too sensitive to other factors than fecal material and may sometimes be misleading, as a test for actual fecal pollution.

Another factor to be considered in the interpretation of direct differential counts of colon-aerogenes organisms, lies in their value as clews to the solution of special problems of water analysis, such as growths in washers and in wood, when such materials are in direct contact with the water. These growths of *Bact. coli* and *Bact. aerogenes* are very disturbing and are often misleading to those who must pass on the findings of sanitary water analysis. Having in themselves no significance of direct fecal contamination, they nevertheless give the findings of fecal pollution in the water.

Table 1 illustrates the value of the differential plate counts in such special problems of water analysis. It shows:

a. An infection of a wooden tank with *Bact. aerogenes*.

b. Infection of a cloth filter with a pure culture of *Bact. aerogenes*.

c. Infection of a fiber gasket and graphited string packing with *Bact. aerogenes*.

d. A group of wells found to have washer growths, in some of which the original suggestive findings were confirmed by examination of the washers themselves, and in some of which the water became negative after chlorine disinfection of the washers.

Such growths are suggested when the differential plate count

TABLE 1

Instances of pumping system infections indicated by differential plate counts

	INDEX PER 100 CC.				INDEX PER 100 CC.		
	Differential plate counts		Standard method		Differential plate counts		Standard method
	Coli	Aerogenes			Coli	Aerogenes	
Wooden tank	0	12	4	At tank influent	0	2	4
Cheese cloth bag on pump nozzle	0	2,647	1,000	5 months after removal	0	0	0
Fiber gasket and graphited string packing	0	2,444	1,000	After treatment	0	0	10
Leather washer	0	66	4	After treatment	0	0	0
	0	92	0				
	0	1,200	1,000	After treatment	0	0	4
	5,000	200	10,000				
	0	246	10	After treatment	0	0	0
	0	32,600	1,000	After treatment	2	0	4
	0	22	6	Aerogenes found in washer			
	242	20	1,000	Aerogenes found in washer			
	70	522	1,000	Aerogenes found in washer			
	0	28	10	Aerogenes found in washer			
	386	3,700	1,000	Abundant coli, few aerogenes found in washer			

shows one of the two organisms predominating almost to the exclusion of the other. *Bact. aerogenes* is the organism most frequently encountered in this connection.

SUMMARY

A study of 147 samples of water from private wells, collected in rural districts adjacent to Chicago, showed a close correlation between the separate count of *Bact. coli,* and the presence of probable sources of fecal pollution as indicated by the sanitary survey. The separate *Bact. aerogenes* count and the colon-aerogenes index by the standard method (American Public Health Association, 1925) also were uniformly positive in this class of sanitary surveys.

The *Bact. aerogenes* counts in the absence of *Bact. coli* were grouped mainly with doubtful sanitary surveys, in which potential rather than imminent danger of fecal pollution was indicated. The standard method indices were also uniformly positive in this group of sanitary surveys.

The colon-aerogenes index as determined by the standard method (American Public Health Association, 1925) was found to be regularly positive in the groups of unsatisfactory and doubtful sanitary surveys, but was also positive in 26.9 per cent of the wells having satisfactory surveys, in which thorough and repeated search failed to disclose any plausible source of fecal pollution. The differential plates from these samples gave no colon-aerogenes colonies.

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THE ELECTROPHORETIC POTENTIAL OF RHIZOBIUM MELILOTI[1,2]

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It is generally accepted that strains of *Rhizobium meliloti* vary greatly in their ability to "fix" nitrogen. The species was divided into two serological groups by Stevens (1923). These groups, designated as A and B, also differ in other respects. Group A grows faster, produces a more abundant growth, is more sensitive to acid, and produces a greater change in titratable acidity and hydrogen-ion concentration of the medium than group B. Stevens (1925) reported that "in general the group A strains fixed almost twice as much nitrogen as did the group B strains." In view of these well defined and constant differences Stevens (1925) appears to be justified in his conclusion that "these variations are manifestations of inherent differences in the nature of the organisms." It seemed logical to us to assume that the "inherent differences" are of such a physico-chemical nature that they should be measurable by suitable methods. This suggested the possibility that differences in electrophoretic potential might exist in relation to the differences in nitrogen fixing ability. A direct relationship between the magnitude of electrophoretic potential and the degree of virulence of pneumococci was reported by Falk, Gussin and Jacobson (1925). During the progress of the present study,

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[1] The experimental data obtained by the Northrop-Kunitz method are from a thesis submitted by R. I. Ferguson in partial fulfillment of the requirements for a degree of Master of Science in the Graduate School of The Pennsylvania State College, 1928.

[2] Published by permission of the Director of the Pennsylvania Agricultural Experiment Station as Technical Paper No. 541.

Jensen and Falk (1928) reported an inverse relationship between the electrophoretic potential and the toxigenicity of diphtheria bacilli.

The purpose of this investigation was to determine, first, if strains of *Rhizobium meliloti* vary in electrophoretic migration velocity and, second, in case variations were found, to note any relationship to nitrogen fixing ability. A preliminary report by Tittsler and Lisse (1928b) indicated a direct parallelism between electrophoretic migration velocity and nitrogen-fixing ability.

Zucker (1929) in a preliminary report of a similar study stated "that a certain relationship exists between acid production and electrophoretic velocity. In general the acid formers show a higher velocity than the non-acid producers. Usually the higher the velocity the poorer the cultures are with respect to their effect on the growth of the higher plant." Later, Zucker, Baldwin and Fred (1930) stated "there are only slight differences in the electric charge carried by various strains of the legume nodule bacteria, and these variations are not correlated with the ability of particular strains to fix nitrogen." Baldwin (1930) stated that "with the alfalfa group the good strains generally have a higher velocity than the poor strains and that just the opposite occurred with the soy bean group. With the other strains studied, it was impossible to make any correlation between the two points. In all cases the differences between strains were very slight."

Zucker (1930) came to the conclusion that "in the alfalfa group, the 'good' strains generally have higher velocities than the poor strains." He showed that the rate of migration velocity was altered by the medium upon which the organisms were grown, the degree of dilution of the cell suspension, the gum or slime and acid by-products, and the storage of the organisms in distilled water.

TECHNIQUE

Cultures. A total of 20 cultures was used in this study. With the exception of strains 1, 2 and 16 they were obtained from other investigators. Strains 5, 6, 7 and 8 were from the group studied by Stevens (1925) who found considerably more nitrogen fixed in plants inoculated with strains 5 and 8 than in those inoculated with strains 6 and 7. Detailed information as to source, original designation and previous descriptions of all cultures is given in table 1.

Medium. The composition of the medium used was as follows:

Potassium phosphate, K_2HPO_4	1.0 grams
Calcium carbonate, $CaCO_3$	2.5 grams
Mannitol, $C_6H_8(OH)_6$	10.0 grams
Agar, Difco	20.0 grams
Distilled water	1,000 cc.

It was tubed, sterilized at 15 pounds pressure for twenty minutes and slanted.

Preparation of suspension. The agar slants were inoculated from fresh cultures grown on the same medium and were incubated for one week at room temperature. The growth was removed with a small volume of distilled water by a gentle rotary movement of the culture tube. This suspension was filtered through cotton, sedimented in a centrifuge at about 3,000 r.p.m. and the cells resuspended in distilled water. The washing of the cells was repeated twice more to remove traces of medium and metabolic products which may have been carried over from the agar. All suspensions were diluted to an arbitrary standard of turbidity and used for electrophoretic measurements as soon as possible on the same day they were prepared.

Measurements of electrophoretic velocities. Migration velocities in the Northrop-Kunitz (1924) cell were measured in essentially the same manner described by Tittsler and Lisse (1928a). Ten measurements of velocity (5 with one and 5 with reversed orientation of the electrical field) were made at 7/16, 8/16 and 9/16 of the depth of the cell. The cell was then refilled and another set of measurements was made. The two sets were averaged provided their differences was not greater than 4 per cent. In case of greater differences, further measurements were made. Thus, each value given represents the average velocity of at least 60 individual bacterial cells.

The capillary method described by Falk, Jensen and Mills

TABLE 1

History of cultures

STRAIN	RECEIVED FROM	ORIGINAL STRAIN	SOURCE	DATE ISOLATED	CHARACTERISTICS OR HISTORY FURNISHED WITH CULTURES
1	Pennsylvania State College	Stock	Alfalfa		Considered efficient
2	Pennsylvania State College		Alfalfa	1927	No plant tests
3	Michigan State College	97			
4	Iowa State College	8	Alfalfa	1925	"Gave positive inoculation"
5	University of Wisconsin	100	Alfalfa	1912	High nitrogen-fixing ability
6	University of Wisconsin	101	Alfalfa	1915	Low nitrogen-fixing ability
7	University of Wisconsin	102	Alfalfa	1916	Low nitrogen-fixing ability
8	University of Wisconsin	107	Alfalfa		High nitrogen-fixing ability
9	United States Department of Agriculture	225	Sweet clover	1914	Low nitrogen-fixing ability
10	United States Department of Agriculture	436	Sweet clover	1922	High nitrogen-fixing ability
11	United States Department of Agriculture	450	Alfalfa	1925	High nitrogen-fixing ability
12	University of Idaho		Alfalfa		"From root covered with nodules"
13	University of Idaho	Stock	Alfalfa		"A very efficient culture"
14	University of Illinois	A. R.			
15	University of Illinois	M.			
16	Pennsylvania State College		Alfalfa	1928	Average nodule production
17	University of Maryland	3DOa10	Alfalfa	1925	"Gave good inoculation"
18	University of Maryland	3DOa9	Alfalfa	1924	"Gave very good inoculation"
19	University of Maryland	3DOa2	Alfalfa	1924	"Gave positive inoculation"
20	University of Maryland	3DOh1	Sweet clover	1924	"No plant tests"

(1928) was used in the second portion of the study to determine whether the earlier results could be duplicated with another type of apparatus. Since the migration velocity in this cell is affected by the length and bore of the capillary, 3 capillaries which gave almost identical velocities in preliminary tests were selected for use in this study. Measurements were made upon 10 bacterial cells (5 with each polarity of the electrical field) in each of the 3 capillaries and the velocities of the 30 cells were averaged.

TABLE 2

Migration velocities and history of all cultures

Type of electrophoresis apparatus

NORTHROP-KUNITZ			FALK CAPILLARY		
Strain	μ/sec. (60 volts)	Nitrogen-fixing ability or history	Strain	μ/sec. (45 volts)	Nitrogen-fixing ability or history
8	70.28	High	5	15.35	High
10	62.24	High	8	15.96	High
11	60.00	High	17	17.05	"Good inoculation"
7	53.23	Low	13	17.08	"Very efficient culture"
5	52.40	High	19	17.10	"Positive inoculation"
1	51.17	Unknown	4	18.25	Unknown
6	50.33	Low	20	18.36	Unknown
3	48.71	Unknown	18	18.82	"Very good inoculation"
4	45.66	Unknown	16	19.24	Average inoculation
2	43.18	Unknown	10	19.38	High
9	35.00	Low	7	20.20	Low
			14	20.43	Unknown
			9	20.58	Low
			15	20.73	Unknown
			12	23.91	"Many nodules"
			6	25.26	Low

The results of our investigation are expressed in terms of observed electrophoretic velocity (in micra per second) at 60 volts with the Northrop-Kunitz cell and at 45 volts with the Falk capillary cell.

EXPERIMENTAL RESULTS

The results of all electrophoretic measurements are summarized in table 2. The cultures are listed in their order of decreasing electrophoretic velocity as determined by the two methods.

Attention is called to the fact that the observed velocities reported for the Falk capillary method vary inversely with their true electrophoretic velocities. With the Northrop-Kunitz method the fastest velocity (70.28μ/sec.) was 100 per cent greater than the slowest (35.0), and with the Falk capillary method the fastest (25.26) was 64.5 per cent greater than the slowest (15.35).

Table 3 summarizes the results obtained with the cultures of known comparative nitrogen-fixing abilities. With the exception of strain 5 in the Northrop-Kunitz method, the high nitrogen-fixing strains had a greater negative electrophoretic potential than those of lower nitrogen-fixing ability.

TABLE 3

Migration velocities or strains of known nitrogen-fixing abilities

Type of electrophoresis apparatus

NORTHROP-KUNITZ			FALK CAPILLARY		
Strain	μ/sec. (60 volts)	Nitrogen-fixing ability	Strain	μ/sec. (45 volts)	Nitrogen-fixing ability
8	70.28	High	5	15.35	High
10	62.24	High	8	15.96	High
7	53.23	Low	10	19.38	High
5	52.40	High	7	20.20	Low
6	50.33	Low	9	20.58	Low
9	35.00	Low	6	25.26	Low

The depressing effect of calcium chloride upon the migration velocity was shown in a single experiment. A suspension of strain 10, prepared as described above, was divided into two lots; to one was added sufficient calcium chloride to make a 1/110 M concentration while the other remained as a distilled water control. The migration velocity in the calcium chloride menstruum was reduced to approximately one-fourth that in distilled water.

DISCUSSION

In view of the fact that various strains of *Rhizobium meliloti* differ greatly in their rate, type and amount of growth, acid-producing ability, sensitivity to hydrogen ions, serological reactions and ability to fix nitrogen in the host plant it is not surprising that they should also differ in their electrophoretic behavior as shown in table 2. The differences of 10 to 100 per cent in the migration velocities are interpreted as expressions of actual differences in the physico-chemical nature of the various strains. The accuracy of the methods has been tested many times in this and other investigations, using suspensions of *Rhizobium meliloti*, *Salmonella pullorum*, and *Escherichia coli*, and we agree with Falk, *et al.* (1925), (1928) and Chapman (1929) that, with uniform technique, the experimental error is not greater than 4 per cent.

A possible relationship between electrophoretic potential and nitrogen-fixing ability is suggested by a comparison of the migration velocities of the known high and low nitrogen-fixing strains (table 3). Both the Northrop-Kunitz and the Falk capillary methods showed that the high nitrogen-fixing strains (with the exception of strain 5, Northrop-Kunitz method only) had a greater negative electrophoretic potential than those of lower ability. Furthermore, with the Northrop-Kunitz method the average velocity (61.64μ/sec.) of the good strains was 33.4 per cent greater than the average (46.19) of the poorer strains, and with the capillary method (velocity inverse to potential) the average (16.9μ/sec.) of the good strains was 30.2 per cent less than the average (22.0) of the poorer strains. The general similarity of the sequences of decreasing electrophoretic potentials obtained by the two methods is rather significant in view of the fact that the capillary method was used by different persons eighteen months after the Northrop-Kunitz method.

Zucker (1930) in analysing his results emphasizes the fact that the variations among different strains were small, never over two micra per second. However, when his differences are considered in terms of percentages rather than as micra per second they appear more significant. Although his fastest velocity (5.14μ/sec.) was only 1.66μ/sec. more than the slowest (3.48) it was 47.5 per cent greater. Furthermore, the average velocity (4.63μ/sec.) of his four known "good" strains was 20 per cent greater than the average (3.86) of the five "poor" ones, although the actual difference was only 0.77μ/sec. With one exception, which might be classified as an intermediate, the "poor" strains had a lower potential than the "good" ones. The validity of our assumption that these differences are significant when considered in terms of percentage is nullified in case an experimental error of equal magnitude is admitted. However, our experience with the Northrop-Kunitz and Falk capillary methods indicates that differences of 10 per cent or more are greater than the experimental error. Therefore, we consider the differences in the "true" velocities observed by Zucker at the "stationary levels" in the Falk (1928) slide cell as signifying actual differences in the electrophoretic potential of various strains.

The observations of Zucker and ourselves that, in general, the strains of higher efficiency had a greater negative electrophoretic potential than those of lower ability were not sufficiently extensive to warrant a final conclusion. Nevertheless, they do indicate a possible relationship which is certainly worthy of further investigation involving a greater number of strains, plant tests and nitrogen determinations. Such a study should also involve a comparison of electrophoretic potential and infectiveness (nodule-forming ability) since Allen and Baldwin (1931) assume that this variable physiological activity is unrelated to effectiveness. In view of the influence of plant passages on the effectiveness of Rhizobia (Allen and Baldwin) it would be interesting to determine if a corresponding change in electrophoretic potential occurs in the course of plant passages. In experiments of this nature, soil conditions must be carefully controlled in view of the fact that certain ions which affect the electrophoretic potential of bacteria may also influence infectiveness and effectiveness.

SUMMARY

1. *Rhizobium meliloti*, like other bacteria, when suspended in distilled water has a negative electrophoretic potential.

2. Variations as great as 100 per cent in the migration velocities of various strains were observed.

3. A relationship between electrophoretic potential and nitrogen-fixing ability is suggested.

4. In general, the order of the results obtained with the Northrop-Kunitz electrophoresis method was duplicated with the Falk capillary method.

5. Calcium chloride, in 1/110 M concentration, had an enormous depressing effect upon the electrophoretic migration velocity.

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